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INTERACTION OF tRNA 3'-TERMINAL NUCLEOTIDYL TRANSFERASE WITH POLYNUCLEOTIDES AND AROMATIC COMPOUNDS

by

© JOANNE I. McCALLA

A THESIS

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The undersigned certify that they have read, and recommend to the Faculty of Graduate Studies and Research, for acceptance, a thesis entitled INTERACTION OF tRNA 3'-TERMINAL NUCLEOTIDYL TRANSFERASE WITH POLYNUCLEOTIDES AND AROMATIC COMPOUNDS submitted by JOANNE I. McCALLA in partial fulfilment of the requirements for the degree of Master of Science.

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ABSTRACT

A study of the interaction of E. coli tRNA 3'-terminal nucleotidyl transferase with various polynucleates and with some small molecules was undertaken, with a view to determining some aspects of the mechanism by which the enzyme recognizes the RNA substrate. It was found that this E. coli enzyme preparation acts in a specific manner, catalyzing incorporation of CMP, but not of AMP, into tRNA lacking two 3'-terminal residues and of AMP, but not of CMP, into tRNA lacking one 3'-terminal residue. Of the polynucleates tested, only tRNA is a substrate; however, non-substrate RNA's can inhibit the reaction of transferase with tRNA and can bind to the enzyme. These complexes are unable to proceed beyond this first non-specific stage to catalysis or even to the more specific type of binding that permits tRNA to protect the enzyme against thermal inactivation. Binding studies with synthetic homopolymers suggest that the binding capacities of polynucleates are inversely related to tendency toward base stacking. Heat denatured tRNA, which is expected to have more base stacking than native tRNA, has less binding capacity and also diminished acceptor activity in the enzymatic reaction.

Study of the effects of polycyclic dyes on this system indicates that they inhibit the transferase reaction by association with the tRNA substrate. The mode of inhibition does not involve intercalation, nor is it merely ionic in character. A positive charge is necessary for effective inhibition but not sufficient. Inhibitory powers within a series of dyes are related to the relative tendencies of the dyes to base stack: the greater the stacking tendency of the

heterocycle, the more effective it is as an inhibitor. Fluorescence data support this hypothesis, providing evidence that the greater the stacking tendency of the polynucleate, the greater the association with acridine orange. There is also indication that bulky substituents on the dyes decrease inhibitory power. These polycyclic dyes inhibit the first stage of the transferase reaction, causing a reduction in the binding of RNA to enzyme.

Polyamines stimulate transferase activity at low concentrations and depress it at higher concentrations. The more cationic species are more stimulatory, and the effects are additive, suggesting that, in this system, polyamines are not mere replacers of Mg^{+2}. The inhibitory effect of polyamines is due to association with tRNA which, at very high concentrations (10 mM), causes precipitation of the RNA. tRNA protection of transferase from thermal inactivation declines steadily as spermine concentration increases, indicating that the stimulatory effect observed in the transfer reaction is not due to an influence on tRNA-enzyme association, prior to the binding of the nucleoside triphosphate substrate.

At a very low concentration ATA inhibits the transferase reaction by association with the enzyme. It inhibits the binding of both R17 RNA and tRNA to transferase and thus represents a probe of the non-specific nucleic acid binding site(s) on transferase. Other triphenylmethanes are also inhibitors but appear not to be so specific as ATA: there is a 40-fold difference in inhibitory power between ATA and the next most effective inhibitor in this series. The specificity, then, of ATA renders it very attractive as a probe of an enzymatic recognition site for transfer RNA.

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LIST OF ABBREVIATIONS

A	adenosine
C	cytidine
G	guanosine
U	uridine
AMP	adenosine-5'-monophosphate
CMP	cytidine-5'-monophosphate
UMP	uridine-5'-monophosphate
RNA	ribonucleic acid
tRNA-pX	tRNA lacking the three 3'-terminal nucleotides (2 CMP + 1 AMP)
tRNA-pXpC	tRNA lacking the 3'-terminal adenosine and cytidine
PP_i	inorganic pyrophosphate
BME	β-mercaptoethanol
s.v.tRNA s.v.R17 RNA	RNA treated with snake venom phosphodiesterase as in Materials and Methods
[^{3}H]CMP-tRNA	s.v.tRNA reacted with [^{3}H]CTP in the presence of transferase and isolated as a radioactive polynucleate
[^{14}C]s.v.R17 RNA	radioactive R17 RNA treated with snake venom phosphodiesterase
R17 RNA-pC	R17 RNA treated with two cycles of periodate-amine cleavage
tRNA-pXpCpC$_{oxi-red}$	tRNA-pXpCpC treated with periodate and borohydride
poly-A	poly-adenosine monophosphate
poly-U	poly-uridine monophosphate
poly-C	poly-cytidine monophosphate
poly-G	poly-guanosine monophosphate

LIST OF ABBREVIATIONS (Continued)

poly-AU	mixed polymer of adenosine and uridine
poly-CU	mixed polymer of cytidine and uridine
ATA	aurin tricarboxylic acid
AO	acridine orange
SDS	sodium dodecyl sulfate
CTA-Br	hexadecyl-cetyltrimethylammonium bromide
S30	fractions of transferase in the purification procedure
S120	
PS11	
B	
DC	
DEAE	diethylaminoethyl
TCA	trichloroacetic acid
NTP	nucleoside triphosphate
c.p.m.	counts per minute
I-50	a concentration of inhibitor sufficient to cause 50% inhibition of the transferase reaction

CHAPTER 1

INTRODUCTION

Some of the first cell-free studies of nucleotide incorporation into polynucleotide material were performed using crude homogenates from various mammalian tissues. Strangely, these systems showed that AMP was incorporated more readily than the other nucleotides, as for example in homogenates of pigeon liver (1), rat liver (2,3), and Flexner-Jobling carcinoma tumour (4). Moreover, it soon became clear that the AMP incorporation occurred adjacent to cytidine residues only. Like other nucleotide-polymerizing reactions, the nucleoside triphosphate was found to be the substrate (5). However, this incorporation was regarded as anomalous because it was known that the average base ratios of various ribonucleic acids were close to A:C:G:U = 1:1:1:1 (6). If the incorporation were due solely to net RNA synthesis, it was not logical to have such a high nearest-neighbour frequency for CpA, in preference to the other three neighbouring frequencies.

A partial explanation of the above phenomena became available through a report from Zamecnik's laboratory in 1958 (7). They discovered that an enzyme from Ehrlich ascites cells induced incorporation of CMP and AMP (as well as some UMP) into so-called soluble RNA (tRNA in present terminology). Moreover, CMP and AMP were added in sequence, with AMP being mostly terminal. The reaction was reversible. Using a rat liver enzyme, Goldthwait (8) demonstrated magnesium-dependent AMP acceptance by two sRNA fractions that had been

separated on Ecteola-cellulose. Thus the substrate had at least been defined, through which it was obvious that net RNA synthesis was not being observed. However, the function of this enzymic activity was not understood at this stage.

Part of the solution to this question came from progress that was being made in the chemistry of ribonucleic acids, especially tRNA. Zamecnik and his colleagues was able to establish in 1958 (9) that the intact -pCpCpA-OH 3'-terminus of tRNA is required to permit amino acid binding to the RNA (or aminoacylation as it is now known), and in 1959 (10) that amino acids are bound in ester linkages to the 2'- or 3'-hydroxyl of the ribose moiety on the terminal adenosine. Subsequently, Allen and Schweet found in 1960 (11) that tRNA lacking the terminal nucleotides does not stimulate [^{14}C]-leucine incorporation into hemoglobin. These findings imply that a terminal adenosine on tRNA provides the acceptor site for aminoacylation, which is the first step toward protein biosynthesis. There seemed to be an enzyme which provided for regeneration of the 3'-terminus of tRNA: tRNA 3'-terminal nucleotidyl transferase (E.C.2.7.7.25), referred to as "transferase" in this thesis.

It was then postulated that perhaps the 3'-terminal nucleotide of tRNA was cleaved after each cycle of amino acid transfer in protein biosynthesis. In an effort to test this, *in vivo* studies were initiated in 1962. Scholtissek demonstrated end turnover of rat liver tRNA for the last nucleotide only (12). Herbert's laboratory found RNA terminally labelled with both AMP and CMP in rabbit reticulocyte red cells (13). AMP turnover was also demonstrated in yeast (14,15), in *E. coli* (16,17) and in sea urchin embryo (18). These studies extended

until 1965, by which time it was clear that the turnover rate was too slow to account for protein synthesis. Moreover, in 1966, Holt et al (19) demonstrated that puromycin had little or no effect on AMP and CMP incorporation into the tRNA of intact reticulocytes although it inhibited the rate of leucine incorporation into protein by 98%. This is direct evidence that the two systems are uncoupled in vivo.

Cannon (17) suggested that the observed turnover was essentially a repair mechanism: ribosome-bound RNase II (an exonuclease requiring single strandedness (20)) randomly degrades the tRNA terminus, and this damage is repaired by transferase. This would result in turnover of the terminal AMP and CMP. In this regard, it is interesting to note that in 1964 Zubay and Takanami found that at 20°C, only two residues of the 3'-terminus of transfer RNA are present in an unhindered and single stranded state (21). This mechanism would thus account for turnover in actively metabolizing mature cells.

On the other hand, for the sea urchin embryo, another interesting possibility was proposed (18). Embryonic growth to the 4-cell stage is accompanied by uptake of radioactive phosphorus in the -pCpCpA-OH end of tRNA. Glišin and Glišin suggested that the addition of the -pCpCpA may trigger protein synthesis after fertilization, since all other components are present before that event.

Another basic role of the transferase system is suggested by the results of Altman (22). He found that the precursors of tyrosine tRNA exist as mega-molecules containing more than one copy of the tRNA. The copies seem to lack the usual 3'-terminal -pCpCpA sequence. If this is so, then the cytoplasmic enzyme which completes this sequence is essential to tRNA maturation and thus to protein biosynthesis.

While the above functional studies were proceeding, other investigators were pursuing a basic characterization of the enzyme system. The results summarize as follows. The reaction catalyzed by transferase does not produce net RNA synthesis: the enzyme has little in common with RNA polymerase for example. The pH optimum for all transferase systems studied so far is 9 - 9.5 (23), whereas the pH optimum for net RNA synthesis is 7 - 8.5 (24). Furthermore, the transferase reaction is not sensitive to actinomycin D, as is RNA polymerase (25). There have been other nucleotide-incorporating enzyme systems reported that do not produce net RNA synthesis (26,27), but on close examination they seem to be different from transferase. After the transferase enzymes from various sources began to be purified, several salient features of the system emerged: AMP and CMP incorporating activities co-purified and were sensitive to heat and to trypsin digestion (28,29). In addition to the tRNA product, pyrophosphate was also liberated. Berg's group demonstrated that amino acid accepting activity could indeed be restored to tRNA, which had been inactivated by snake venom phosphodiesterase, by re-addition of the -pCpCpA terminus (30). tRNA that is degraded beyond the terminal three nucleotides cannot accept CMP (30,31). The reversibility of the reaction is not quite complete: for the yeast enzyme, only one CMP is removed by pyrophosphorolysis (the reverse of incorporation), while two can be replaced if removed by other means (32).

Thus the basic reactions that can be observed in this system regardless of the biological origin of the enzyme, are:

$$\text{tRNA-pX} + 2\text{CTP} + \text{ATP} \xrightarrow{Mg^{2+}} \text{tRNA-pXpCpCpA} + 3\text{PP}_i$$

$$\text{tRNA-pXpCpCpA} + 2\text{PP}_i \xrightarrow{Mg^{2+}} \text{tRNA-pXpC} + \text{CTP} + \text{ATP}$$

This system presents a unique opportunity to study protein-nucleic acid interaction. First, the basic processes of the reaction have been well characterized, so that the effects of perturbers of the system can be assessed. Second, it is as simple a system as there is presently available: there is interaction between one of the smallest species of RNA (tRNA) and enzyme, and between two nucleoside triphosphates and enzyme. This makes it a practical model for protein-nucleic acid interaction. A third advantage of considerable importance is the fact that the primary and secondary structures of tRNA's are largely known (see for review, Ann. Rev. Biochem. (33)). Primary sequences have been determined for many tRNA species, and the clover-leaf model for secondary structure seems to account for many of the observed physical and chemical properties of tRNA. It is important to note that transferase is able to interact with all tRNA species. This is in marked contrast to the aminoacyl-tRNA synthetases, each of which is specific to a single tRNA species, under normal conditions. The primary sequences of different tRNA's show no similarity, except in the 3'-terminus, the pseudouridine loop, and the dihydrouridine loop. Therefore, the recognition of tRNA by transferase should involve features common to all tRNA molecules, either in primary or higher structure.

The question thus arises as to how this recognition mechanism can be investigated. One possible approach is kinetic studies. This method has served to demonstrate that there are two separate sites on the enzyme for ATP and CTP. This conclusion was reached for both the rabbit muscle (34) and the E. coli (35) enzymes, although the types of inhibition kinetics were different. However, it

is difficult to extend this method to an investigation of the interaction between transfer RNA and enzyme. First, it is not known whether there is a single site of association with the RNA molecule or multiple sites. This makes interpretation of kinetic data difficult. Another problem is the fact that kinetic experiments generally require homogeneity of the substrates. In this case, the complexity of the tRNA substrates gives rise to difficulty since the enzyme does act on all species of tRNA. Hence conclusions drawn from kinetic experiments using a single amino acid accepting tRNA species cannot be generalized. Conversely, kinetic experiments using mixed tRNA's may represent only an averaged condition.

A second possibility would be to investigate cases where the enzyme "misbehaves" by recognizing incorrect substrates. There have been numerous instances of this type of event, especially in the mammalian systems. These include cases in which the "wrong" nucleoside monophosphate is incorporated into tRNA (23,31,36-41). Depending on the reaction conditions, virtually all mammalian systems can catalyze reactions such as the following:

$$\text{tRNA-pXpC} + \text{ATP} \longrightarrow \text{tRNA-pXpCpA} + PP_i$$

$$\text{tRNA-pXpC} + \text{UTP} \longrightarrow \text{tRNA-pXpCpU} + PP_i$$

$$\text{tRNA-pXpCpC} + \text{CTP} \longrightarrow \text{tRNA-pXpCpCpC} + PP_i$$

Of these products, only tRNA-pXpCpA is able to carry out the normal functions of tRNA (aminoacylation, peptide transfer (31). tRNA-pXpCpU and tRNA-pXpCpCpC are not capable of accepting AMP.

In addition, some transferase systems can recognize polynucleates other than tRNA: Yot et al found that the E. coli enzyme could use as substrate turnip yellow mosaic virus RNA (42). It was then established that this RNA actually contained a tRNA-like structure at the 3'-terminus. However, some mammalian enzymes will catalyze a poly-C polymerase-type reaction, in which lengths of poly-C sequences are added to such species as 5S RNA (38,39). In E. coli some purified preparations do catalyze misincorporation (41), while others do not (43).

There are two major difficulties in interpreting these misincorporation studies. One is the difficulty in determining what permitted the association of the "incorrect" nucleic acid or nucleotide substrate with the enzyme. Was it structural similarity to the normal substrate or some other undefined process? The misincorporation reactions seem to occur more readily in the presence of manganese II (instead of magnesium ion) or in the presence of high enzyme concentrations (38,39,41). Although many of the enzyme preparations are highly purified, none attain complete homogeneity. There is also some evidence that multiple species of transferase exist. Gross et al have resolved their purified E. coli enzyme into two peaks of activity (44). In addition, Mukerji and Deutscher have isolated a mitochondrial transferase from rabbit liver (45) which may correspond to one of the two activities Deutscher had observed earlier (39). It is not at present known whether these multiple transferases behave similarly with respect to misincorporation. In view of these problems, it is difficult to draw conclusions from these data, concerning the mechanism of recognition of transfer RNA by transferases.

A third type of approach is to examine structural aspects of the recognition mechanism via chemical modification of the transfer RNA. Some very useful information has become available through this means. It has been found that tRNA blocked at the 2'(3')-hydroxyl of the ribose moiety on the terminal adenosine can not be pyrophosphorolyzed (46,47). Pulkrabek and Rychlik (47) suggest that the enzyme may require a free cis-hydroxyl configuration at the 3'-terminal ribose. In 1971, von der Haar et al reported that when tRNA-pXpCpC^{phe} was treated first with $NaIO_4$ and then with $NaBH_4$ to remove the C_2'-C_3' bond, thereby destroying the hydroxyl cis-configuration (tRNA-pXpCpC$^{phe}_{oxi-red}$), it could not be pyrophosphorolyzed (48). On the other hand, tRNA-pXpCpCpA$^{phe}_{oxi-red}$ could be pyrophosphorolyzed, and there is no cis-hydroxyl in either of these species. Thus the effect of blocking the 2'(3')-hydroxyl (46,47) is likely due not to loss of the cis-hydroxyl but to bulky substitution at that site. Von der Haar et al explain the different behaviour of tRNA-pXpCpC$^{phe}_{oxi-red}$ and tRNA-pXpCpCpA$^{phe}_{oxi-red}$ on the basis that oxidation and reduction distorts the ribose configuration: this distortion would be different for C_{75} and A_{76}.

Other chemical studies have indicated that transferase is tolerant to modification at the 5'-hydroxyl of the terminal ribose moiety (49) and at the C5-position of a terminal cytidine (50). On the other hand, deamination of a terminal cytidine to uridine causes loss of AMP acceptance (51). This is consistent with the results of Daniel and Littauer which indicated that the misincorporation product tRNA-pXpCpU does not accept AMP (31).

However, studies on the function of the 3'-terminus of tRNA in the recognition process must also take into account another fact.

Namely, neither R17 RNA, which has the 3'-terminal -pCpCpA, nor synthetic polynucleates such as poly-C or poly-AC are substrates of the enzymic reaction. The other invariant sequences of tRNA, in the dihydrouridine and pseudouridine loops, should also be investigated. These other regions of the molecule can be chemically modified, for example, by deamination (52), photo-coupling reactions (53), and excision reactions (54). Unfortunately, there are inherent difficulties in these methods. Many of the available reactions are relatively non-specific. Moreover, most of the relevant sequences in tRNA are protected by secondary or tertiary structure in the native conformation. Thus it is difficult to induce alteration in these regions without massive and undefined modifications elsewhere. This was not a severe problem in the study of the 3'-terminal sequence because, as mentioned earlier, this sequence is largely exposed in a single stranded and unhindered state so that these nucleotides react much more readily than internal nucleotides. Also, of course, there is a specific reaction available for 3'-termini of nucleic acids (i.e., the periodate reaction).

Even if specific reactions can be found, there is also the problem that it is not known what effect modification of the primary structure of tRNA has on secondary and tertiary structure. Thus it is difficult to determine whether a disruption of the recognition process is due to the primary alteration or to undefined secondary and tertiary alterations.

Fragmentation experiments represent a fourth approach to the study of the recognition mechanism. The tertiary structure permits specific fragmentation of tRNA into half-, quarter-, or three-

quarter-molecules by RNase T1 (55). The fragmented tRNA can then be examined for nucleotide accepting activity in the transferase system. Gross et al found that the E. coli enzyme could cause tRNA fragments of chain length 40 to accept AMP (44). Zachau's laboratory published fragmentation results on $tRNA^{ser}$ and $tRNA^{phe}$ from yeast (55). There was about 13% accepting activity for the 3'-half of $tRNA^{phe}$, tested alone. When this was combined with the 5'-half of $tRNA^{phe}$, almost complete recovery occurred. Likewise, this recovery could be almost complete when the 5'-half of $tRNA^{ser}$ replaced that of $tRNA^{phe}$; combining the 3'-half of $tRNA^{ser}$ with the 5'-half of $tRNA^{phe}$ did not result in recovery of activity. Interpretation becomes difficult because a question arises as to the fidelity of the reassociation process. That secondary structure is important to the recognition process was first discovered in 1967, when Lindahl et al investigated a renaturable yeast tRNA-$pXpCpC^{leu}$ and found that AMP incorporation occurred readily when the nucleic acid was in its native conformation but poorly when it had been denatured (56). In 1971, Igarashi and McCalla reported a similar phenomenon for CMP incorporation into bulk E. coli tRNA (57). Therefore a negative result in fragmentation experiments is often impossible to interpret. Another limitation of this method is the difficulty of examining the first step of the recognition process (binding of the tRNA to the enzyme). It is known that there is possible non-specific interaction between enzymes and various types of RNA's, as demonstrated in the aminoacyl-tRNA synthetase system (58). Thus it is not useful to attempt binding studies using fragments of tRNA. It seems that a necessary type of investigation to pursue would be a general study of structural

requirements, including where possible those related to primary sequence of tRNA.

A fifth approach is to investigate enzymic thermal stability changes due to binding of substrate. This method is particularly useful in isolating the events of the transfer reaction, as these relate to the tRNA substrate. On a gross scale, the first event will be binding of the tRNA to the enzyme, then (perhaps) a conformational change in enzyme and/or tRNA, and finally the actual catalytic event--incorporation of the mononucleotide into tRNA. Each of these steps is amenable to study, via a combination of the above approaches.

Binding of tRNA to enzyme can be measured in various ways. Among the methods that have been used are sucrose density gradient centrifugation (59) and millipore filtration (57,60). Of these, the millipore method is the less harsh process, capable of detecting less stable complexes than the other. However, the sensitivity of the filtration method does depend on experimental detail so that comparison among results of different groups is difficult, as will be discussed in Chapter 3.

Whether observed binding represents catalytically important binding can be determined in two ways. If the substance binds in the catalytic site in precisely the way a normal substrate does, then one could expect incorporation to proceed. If, however, the binding substance is not a substrate as determined by incorporation, it may still bind in an enzymatically important fashion. If it does so, then one can expect that it will inhibit the normal incorporation reaction using the tRNA substrate (57).

A substance may do all of this and yet not proceed beyond this first stage of the transfer reaction. One measure of whether the reaction proceeds beyond this stage is determining if the binding substance provides protection against thermal inactivation of transferase. As mentioned earlier, transferase is sensitive to heat. It can be protected from inactivation by the presence of tRNA or the nucleoside triphosphates (43,57). Interestingly enough, Miller and Philipps have found that acylated tRNA is able to protect the E. coli enzyme, in spite of its not being a substrate for the transfer reaction (46,47). Extensive digestion of tRNA with snake venom phosphodiesterase, especially after removal of about 9 nucleotides from the 3'-terminus decreases ability to protect the enzyme. The 3'-terminal neck appears to have considerable importance in the recognition process. In 1971, Chapeville's laboratory succeeded in producing a sepharose-bound transferase from E. coli which had greater heat stability than the soluble enzyme (61). However, tRNA failed to increase that stability as it does for the soluble enzyme. These somewhat random points may serve to indicate some of the scope of thermal inactivation studies.

In this thesis, the methods used were the four basic types of study discussed immediately above:

1. Incorporation of mononucleotides into tRNA (E. coli)
2. Binding of polynucleates to transferase (E. coli)
3. Inhibition of the transfer reaction by "binding substances"
4. Thermal inactivation

The aim was to determine some aspects of transferase recognition of its substrate nucleic acid. Each tool was used as a probe of the appropriate stage of the reaction as outlined above. Incorporation measures the sum of the stages, binding the first stage, and protection against thermal inactivation all those stages that precede actual catalysis. Inhibition by the "binding substances" is a test of the type of binding. In addition to these basic studies, the methods were also used to assess the effect of perturbers of the system: polycyclic dyes, triphenylmethane dyes, and polyamines.

CHAPTER 2

MATERIALS AND METHODS

Chemicals

The following radioactivaly labelled chemicals were purchased from Schwarz/Mann: [^{3}H]CTP (20.4 Ci/mmole), [^{3}H]ATP (21.7 Ci/mmole), [^{3}H]poly-A (6.15 mCi/mmole polynucleotide phosphorus), [^{3}H]poly-U (7.76 mCi/mmoleP), [^{3}H]poly-C (6.97 mCi/mmole P), [^{3}H]poly-AU (8.8 mCi/mmole P), [^{3}H]poly-CU (7.5 mCi/mmole P), [^{14}C]adenine (52.6 mCi/mmole), [^{14}C]uracil (60 mCi/mmole). CTP and ATP were also from Schwarz/Mann. [^{3}H]poly-G (31.7 µCi/mmole P) was obtained from Miles Laboratories Inc. Proflavine sulfate, acridine orange, and ethidium bromide were from Calbiochem, while 9-aminoacridine and fluorescein were obtained from Sigma. The other polycyclic dyes, as well as all the triphenylmethane dyes except ATA (from Fisher Scientific Co.) were Baker products. L-lysine monohydrochloride, Norit-A, phosphodiesterase from Crotalus adamanthus venom (type II), and alkaline phosphatase were from Sigma. The polyamines were obtained from Mann Research Co. Hexadecylcetyltrimethylammonium bromide was obtained from Eastman Organic Chemicals. Tripticase Soy Bean Broth was a product of Baltimore Biological Laboratories. All other chemicals were reagent grade from Fisher Scientific Co.

Biological Materials

E. coli tRNA was purchased from Schwarz/Mann. R17 viral RNA was extracted from virion according to the method described earlier (62). Briefly this was as follows:

E. coli was grown in TM-medium (in grams per liter: 6.05 Tris, 5.8 maleic acid, 2.5 NaCl, 2.0 KCl, 1 NH_4Cl, 0.142 Na_2HPO_4, 0.142 Na_2SO_4, and 0.025 $MgSO_4 \cdot 7H_2O$; the pH was adjusted to 7.3 with 5 N NaOH). TM-medium was supplemented with 1 ml of 50% glucose and 0.5 ml of 0.25% L-methionine per 100 ml. The cells were grown in two steps. First, a culture medium was incubated with E. coli K12 Hfr met- and incubated overnight at 37°C in a rotary shaking water bath. Then 0.2 ml of the overnight culture was reinoculated into 50 ml of fresh culture medium, and allowed to grow at 37°C for 3 hr with shaking. Fifteen minutes before infection, the shaking of the water bath was reduced to minimize breakage of F-pili from the host cells. Phage R17 was added to the culture at a multiplicity of infection of 20. Shaking was increased to moderate speed after 10 min of infection. 120 min after infection, NaCl was added to a final concentration of 0.1 M, and this was followed immediately by the addition of 150 ml of ethanol. This suspension was kept in the cold overnight, after which the precipitate was collected by centrifugation. Phage particles were purified from the pellet by successive washing with buffer (50 mM Tris-HCl, pH 7.4--1M KCl--1mM EDTA) using high speed ultracentrifugation (150,000 XG for 90 min). RNA was then extracted by the sodium dodecyl sulfate (SDS)-phenol method (63).

When radioactively labelled R17 RNA was required, an appropriate amount of [^{14}C]adenine and [^{14}C]uracil was added at 20 min after infection. Phage were harvested by the standard method, and viral RNA was extracted as above.

Preparation of RNA Free of 3'-terminal Adenine and Cytidine Residues

Method A

This procedure involves stepwise degradation by a modified periodate method (64). 50 mg of tRNA was dissolved in 1.0 ml of 0.2 M NaCl and put into a round-bottom centrifuge tube with a stirring magnet 1/8 x 3/8 in. A 2.5 ml aliquot of 0.1 M hexadecylcetyltrimethylammonium bromide (CTA-Br) was added slowly at room temperature with constant stirring. After two additional minutes of mixing, the magnet was removed, and the insoluble CTA-tRNA complex was spun out at 10,000 r.p.m. for 10 min using a type JA20 rotor in a Beckman J21 centrifuge at room temperature. The precipitate was dissolved in 1.5 ml 1 M lysine buffer (pH 8.5), and subjected to periodate treatment as follows. A 0.5 ml aliquot of 2 M lysine-HCl (pH 8.5) and 0.5 ml 30 mM periodate were added, and the mixture was incubated in the dark at 45°C for 2 hr. After cooling to room temperature, a 10-fold molar excess of ethylene glycol was added to quench unreacted periodate. Water was then added to precipitate the RNA-CTA complex by reducing the chloride concentration to less than 0.2 M. After centrifugation, the pellet was washed with 0.2 M NaCl--0.01 M CTA-Br and was redissolved in 1 M NaCl--0.2 M lysine-HCl (pH 9)--0.005 M ethylene glycol. Alkaline phosphatase (10 units) was added and the solution incubated at 37°C for 3 hr. The RNA was then precipitated,

dissolved in 1 M NaCl, reprecipitated, and finally dissolved in 1 M lysine-HCl (pH 8.5) in readiness for another cycle of treatment. If sufficient cycles of treatment had been performed, 1/100th volume of 1 M $MgCl_2$ and 2 volumes of ethanol were added after the incubation with phosphatase, and the tRNA was collected by centrifugation. The tRNA free of CTA thus obtained was washed with a 1:1 mixture of ether and ethanol and with ether. After the dried precipitate had been dissolved in water, the tRNA was ready for use in the assay system.

When R17 RNA was treated with periodate, the same conditions and proportions as above were used.

Method B

This method is based on limited digestion of the 3'-terminus of RNA by snake venom phosphodiesterase. tRNA (50 mg) and 0.045 unit of phosphodiesterase were incubated in 1 ml of 0.1 M lysine-HCl (pH 8.5)--1.2 mM $MgCl_2$ at 37° for 30 min. The reaction mixture was extracted three times with 1 ml of phenol, and the phenol layers were re-extracted with water. The water layers were then pooled and precipitated by the salt-ethanol method. Precipitation by ethanol without prior phenol extraction did not destroy all the snake venom phosphodiesterase activity. The precipitate was washed with ether-ethanol and ether, dried and dissolved in water. These conditions result in tRNA which can accept CMP, but not AMP, in the transferase reaction. This fact suggests that, on the average, not less than two residues were removed from the 3'-terminus of tRNA. tRNA treated as above is designated s.v.tRNA in this thesis.

[^{14}C]R17 RNA (and unradioactive R17 RNA) free of the 3'-terminal residue(s) was prepared by this method at the same enzyme-substrate ratio in terms of moles. Paper chromatography (as outlined below) of the released radioactive nucleoside(s) revealed that adenosine was the major product. It appears that the 3'-terminus of R17 RNA is not as accessible to phosphodiesterase as that of tRNA. [^{14}C]R17 RNA treated as above is designated [^{14}C]s.v.R17 RNA, unradioactive R17 RNA as s.v.R17 RNA.

Tritiated tRNA free of the terminal adenine was prepared by incubating 5.6 mg of s.v.tRNA and 500 µg of RNase-free transferase at 37°C in 20 ml of 0.1 M Tris-HCl or 50 mM glycine-NaOH (pH 9.0)--0.01 M $MgCl_2$--0.001 M mercaptoethanol (βME), to which 0.19 mCi of [^{3}H]CTP had been added. At 60 min, the RNA was precipitated by the CTA method. After being dissolved in 1 M NaCl, it was precipitated by the ethanol-salt method and washed as usual. This tRNA is designated [^{3}H]CMP-tRNA.

Desalting and Identification of Bases and Nucleosides

Supernatant solutions containing bases or nucleosides released from RNAs by either of the methods given above were desalted and chromatographed. A method for desalting bases and nucleosides using activated charcoal (Norit-A) was developed. Norit-A was washed prior to use as follows: (1) three times with water, (2) three times with 9 M formic acid, (3) three times with 9 M NH_4OH, (4) three times with isopropanol-NH_4OH-H_2O (9:6:5), and (5) with water until the effluent pH was neutral. A small amount (0.3 g) of this washed charcoal was put into a sintered glass funnel which was attached to a vacuum system. The base or nucleoside solution was filtered through

the charcoal under vacuum and washed with water to remove salts. The retained base or nucleoside was eluted with a mixture of isopropanol-NH_4OH-H_2O (9:6:5). This method gave over 90% recovery of all the common nucleosides and bases. This effluent was collected and flash-evaporated. The residue was dissolved in 1 M acetic acid or 0.1 M HCl and spotted on Whatman No. 1 paper that had been impregnated with 1 M $(NH_4)_2SO_4$. The chromatogram was run using as solvent a mixture of ethanol-H_2O (8:2) (65).

Periodate treatment of E. coli tRNA resulted in the release of adenine in the first cycle and about 61% cytosine and 39% adenine in the second. The amount of adenine released in the first cycle per milligram RNA is approximately equal to the amount of cytosine released in the second cycle per milligram RNA ($A_1/C_2 = 1.08$). This suggests that cytosine is removed in sequence after adenine, in keeping with the fact that the 3'-terminal sequence of tRNA is -pCpCpA-OH. The adenine released in the second cycle may be the result of periodate removal of an internal residue which is accessible to periodate because of a nick in the chain. This removal may or may not affect the acceptor activity of the RNA molecule. However, a large excess of RNA is always used in the assay mixtures so that these few, possibly inactive, species would be undetectable.

Isolation of Transferase

E. coli K-12 was grown in Tripticase Soy Bean Broth medium, and harvested by continuous-flow centrifugation. The soluble proteins were obtained as described earlier (66). The harvested cells were stored at -20°C until used. They were then thawed and suspended

in 1 ml of buffer-A (0.01 M Tris HCl (pH 7.8)--0.010 M $MgCl_2$--0.006 M β-mercaptoethanol) per mg of cells. Two micrograms of DNase [E.C.3.1.4.5] were added for each ml of suspension. The cells were disrupted by sonic oscillation for 5 min at 1 KC per sec. The resulting suspension was clarified by centrifugation at 30,000 x g for 15 min (S30 fraction). Any pellet was resuspended in buffer-A, re-sonicated, and re-clarified. The supernatants were combined and clarified by ultracentrifugation at 120,000 x g for 90 min.

The supernatant proteins (S120 fraction) were adjusted to 20 mg/ml and 0.02 mg of protamine sulfate in solution was added dropwise for each A_{260} unit. Fifteen minutes later, the precipitate was collected by centrifugation at 30,000 x g for 10 min and suspended in buffer-A. After being allowed to stand at 4°C overnight, the precipitate was collected by centrifugation (10,000 r.p.m., JA20 rotor, Beckman J21 centrifuge). The precipitate was then resuspended in buffer-A containing 0.1 M KCl. The transferase-containing supernatant fraction (PS II) was then subjected to batchwise chromatography on DEAE-cellulose (Carl Schleicher and Schuell Co.). Activity occurred mostly in the fractions eluted at lower salt concentrations (0.15 M KCl).

Proteins eluted from the column with 0.15 M KCl in buffer-A were precipitated at 4°C with ammonium sulfate at saturation concentration (B fraction), then dialyzed overnight against buffer-A. After the dialyzed solution was clarified by centrifugation, the supernatant was diluted five times with buffer-A and loaded to a DEAE-cellulose column previously equilibrated with buffer-A. Proteins were eluted by a linear gradient of KCl from 0 to 0.3 M, using an ISCO

U.V. monitoring system. The fractions were assayed for transferase and RNase activity. The transferase assay was done using 2/5 of the standard incorporation system as described below.

The RNase assay was done as described earlier (67). Briefly, the assay was conducted at 37°C in 0.2 ml of reaction mixture containing 0.1 M Tris-HCl (pH 7.8), 0.01 M $MgCl_2$, 0.06 M KCl, 0.006 M βMe, 30 mμCi [^{3}H]poly-U and portions of each fraction. After incubation for 20 min, 150 μl aliquots were spotted on paper discs, and these were subjected to cold TCA washing as described previously (68). In short, the discs were soaked in cold 10% TCA for 40 min, followed by 4 successive 15-min washes with cold 5% TCA. They were then washed with ether-ethanol (1:1) (15 min at room temperature), followed by a final 10-min wash with ether at room temperature. After being dried, the discs were put in toluene scintillation fluid and radioactivity retained on each filter was measured in a Beckman Liquid Scintillation system LS-200B.

The fractions which contained transferase but not RNase activity were pooled and concentrated by adsorption to a small quantity of DEAE-cellulose followed by elution with buffer-HSB (0.1 M Tris-HCl (pH 7.8), 10 mM $MgCl_2$, 6 mM βME, and 500 mM KCl). The condensed enzyme (DC fraction) was stored at -20°C. In later preparations, the condensation was done by dialysis against 50% glycerol in buffer-A.

At the final stage (DC fraction), the extent of purification was approximately 200-fold (from about 50 c.p.m./min/μg protein for the S120 fraction to 10,000 c.p.m./min/μg for the DC fraction). While

this was not a completely homogeneous preparation, it contained no detectable aminoacyl-tRNA synthetase, RNase, DNase, DNA-dependent RNA polymerase, M factor (69), polynucleotide phosphorylase, or phosphatase activity. Thus the major possible enzymic contaminants which have affinity for nucleic acids were not present.

As well, it should be noted that rechromatography of DC fraction on DEAE-sephadex (Pharmacia) and phosphocellulose (Carl Schleicher and Schuell Co.) resulted in no change in specific activity. Thus it was concluded that some means other than ion-exchange chromatography would be required to carry the enzyme purification further. This has in fact been accomplished by Miller and Philipps (43), using hydroxylapatite chromatography.

CMP and AMP Incorporation

The reaction mixture contained in 0.5 ml: 0.1 M Tris-HCl or 0.05 M glycine-NaOH (pH 9), 10 mM $MgCl_2$, 1 mM BME, 50 µg tRNA-pXpC, tRNA-pXpCpC or s.v.tRNA, 5 µg RNase-free transferase, and 4.5 µCi [^{3}H]ATP or CTP. The reaction took place at 37°C for 12 or 40 min. At intervals, 0.1 ml aliquots were withdrawn and placed on filter discs which had been impregnated with 0.1 ml of 2.5 mM CTP or ATP to prevent non-specific adsorption of [^{3}H]ATP or CTP to the discs. These discs were then subjected to cold trichloroacetic acid (TCA) washing as described above. Initial rates were calculated from the linear part of the progress curves.

Binding Experiments

Binding studies were performed with radioactively labelled RNA's. Incubation mixtures contained in 0.2 ml: 20 mM potassium phosphate buffer (pH 5.6), 5 mM βME, 2 µg bovine serum albumin (BSA), 12 µg transferase, and various amounts of radioactive RNA. After 5 min incubation in the cold, samples were diluted 10 times with a buffer containing 20 mM potassium phosphate (pH 5.6) and 5 mM βME, and filtered by suction through membrane filter discs (Millipore, either HAWG or DAWP) (70). The discs were washed first with 2 ml of cold 10 µg/ml BSA in the buffer and then with 10 ml of cold buffer. After drying, the radioactivity retained on each filter was counted in toluene scintillation fluid. Control samples contained no enzyme. The difference in radioactivity between experimental and control sets represented the amount of labelled RNA bound to the enzyme. It should be pointed out that Millipore filters had to be selected according to the criteria of (a) high adsorption of enzyme, and (b) little or no adsorption of RNA in the absence of enzyme. Very few filter lots were satisfactory on this basis.

Thermal Inactivation

Thermal inactivation of transferase was studied at pH 5.6 and 9.0 and at three temperatures: 50°, 55°, and 60°C. A 6 µg sample of RNase-free enzyme in 50 µl of buffer (either 20 mM potassium phosphate (pH 5.6)--5 mM βME, or 100 mM Tris-HCl (pH 9.0)--10 mM $MgCl_2$--1 mM βME) was incubated at the desired temperature. To test for protection of the enzyme from thermal inactivation, various

amounts of polynucleates were added to the pre-incubation mixture. After pre-incubation, the standard CMP-incorporating experiment, as described above, was performed to determine the activity remaining.

Inhibition Studies with Dyes

The incorporation reaction was carried out as outlined above, except that various amounts of inhibitor (polycyclic or triphenylmethane dye) were added to the mixture. Initial rates were determined from the progress curves of the reactions in the presence of varying levels of dye. The enzyme activity at each inhibitor concentration was expressed as a per cent of the activity in the absence of dye.

Pre-incubation Study

Pre-incubation of dye with the various reaction components (s.v.tRNA, enzyme, and a mixture of both) was carried out on ice. Since the enzyme is very heat labile in the absence of tRNA (as described in the introduction, Chapter 1), the pre-incubation could not be carried out at 37°C. After incubation on ice for 5 min, enzymic activity was measured as usual.

Sephadex G25 Chromatography of an Acridine-tRNA Complex

Sephadex G25 (Pharmacia) was pre-equilibrated with 50 mM glycine-NaOH (pH 9)--5 mM $MgCl_2$--1 mM BME. The load solution consisted of one ml of 3.1 mg/ml s.v.tRNA and appropriate amounts of acridine orange (AO) in the same buffer. Elution was with the pH 9 buffer at a flow rate of one ml per min. tRNA concentration in the eluate was determined by the orcinol method (71). The amount of

acridine orange in each fraction could not be directly determined by absorbance measurement because the presence of tRNA in the solution changed the absorbance spectrum of the dye. A portion of each fraction was thus subjected to alkaline hydrolysis (0.3 M NaOH) at 37°C overnight. This resulted in complete degradation of tRNA and restoration of the characteristic visible spectrum of acridine orange. Use of a standard AO solution thus permitted quantitation of the amount of AO in each fraction (λ_{max} AO = 493 nm).

Spectrofluorometric Study of AO-RNA Complexes

All polynucleotides used were precipitated as their potassium salts by the methanol precipitation method, and dried *in vacuo*. A solution of each RNA was adjusted to 10 mg dry weight of this salt per ml of distilled-deionized water. The molar concentration of nucleoside monophosphates was calculated accordingly. The average molecular weight of nucleotide as the potassium salt was assumed to be 380. Thus, the concentration of nucleotide in the tRNA solution was estimated as 2.63×10^{-2} molar.

Acridine orange was purified as follows: the dye was dissolved in 0.01 N HCl and precipitated by adding an equal volume of 0.1 N NaOH. The precipitate was collected by centrifugation and dissolved in chloroform. The dye was then extracted from the chloroform solution with 0.1 N HCl. After one repetition of the above procedure, AO was crystallized from the chloroform solution and dissolved in a minimum volume of 0.1 N HCl. The dye solution was brought to 10 mM, by dilution with water, and its concentration determined by optical methods, assuming that the extinction coefficient of AO at infinite dilution is 56,000 at 492 nm (72).

The solutions to be analyzed were prepared so as to avoid formation of an insoluble complex between AO and RNA. First, 0.25 ml of Tris-HCl (1 M, pH 8) and 4 ml of distilled water were mixed in a 10 ml graduated cylinder. A suitable amount of AO was added to the buffered solution and mixed thoroughly. RNA was then added slowly with vigorous shaking. Finally, by adding distilled water, the volume was brought to 5 ml and used for fluorescence measurements.

Spectrofluorophotometry was performed using a Turner model 210. In order to assess the molecular interaction between AO and various RNA's under conditions resembling those used in the enzymic reaction described earlier (10^{-3} to 10^{-4} M AO), it was necessary to reduce the sensitivity of the spectrophotometer to a minimum. The setting of the equipment was as follows: energy level--10 (instead of 30 for normal operation); excitation slit width--100 Å; emission slit width--25 Å; and sensitivity range--X1. The temperature of sample solutions was maintained at 30°C by a circulated thermoregulator (Haake model FS). The AO-RNA mixture was excited by light at 300 nm, instead of light with wavelength in the visible range. The reason for this unusual choice of excitation procedure was as follows: the apparent quantum yield of AO can be reduced by use of this wavelength, and both the excitation and emission spectra of AO in the visible range shift drastically after complexing with RNA, while the excitation spectrum at 300 nm does not. Thus, excitation at 300 nm enables one to avoid erratic measurement of fluorescence yield in a wide range of AO concentrations. When the concentration of AO was too low to be measured, the sensitivity of the spectrophotometer was increased by changing the sensitivity setting, leaving other settings unaltered.

CHAPTER 3

CHARACTERIZATION OF THE E. COLI TRANSFERASE SYSTEM

Introduction

It has been known for some time that tRNA 3'-terminal nucleotidyl transferase catalyzes two reactions: pyrophosphorolysis of the two 3'-terminal nucleotides in tRNA, and transfer of AMP and CMP to tRNA lacking the terminal residues (29,30). More extensive studies with purified transferase and purified tRNA have revealed the absolute stoichiometry of the transfer reaction:

$$\text{tRNA-pX} + 2\text{CTP} + \text{ATP} \xrightarrow{Mg^{+2}} \text{tRNA-pXpCpCpA} + 3PP_i \quad (32),$$

where X stands for the fourth nucleotide from the 3'-terminus of intact tRNA. The enzyme has been highly purified from various sources, and its enzymic activity shows specificity towards tRNA (32,35,41,60,73). The biological function of the enzyme appears to be the regeneration of an intact 3'-terminus of tRNA for amino acid acceptance (30).

Recent advances in the analysis of the base sequences of macromolecular RNA's have shown that several of these species possess the 3'-terminal sequence, -pCpCpA-OH (74-77). This is identical to the analogous sequence in tRNA. Yet attempts to cause these RNA's to undergo the transferase reaction have failed (78). A notable exception is turnip yellow mosaic virus RNA, where a valine-specific tRNA-like structure seems to occur at the 3'-terminus (42). In addition, Deutscher has demonstrated a poly-C polymerase activity in his purified preparation of transferase from rabbit liver (39). This activity

requires as a primer, rRNA, intact tRNA or tRNA lacking the 3'-terminal residues. It does not act on Qβ-RNA, poly-C, poly-A, or poly-U. However, this is one of the anomalous reactions which only occur at high enzyme concentrations, or in the presence of Mn^{+2} instead of Mg^{+2}. This liver enzyme, as well as the E. coli transferase of Best and Novelli (41), shows incorporation of AMP into tRNA-pXpC in the absence of CTP.

It is known that most tRNA species vary considerably in base sequence, except for three regions: the 3'-terminus (-pCpCpA), the so-called dihydrouridine loop, and the TψCG loop. The fact that other RNA species with the CpCpA-OH-3'-terminus are not normal acceptors of CMP or AMP in this enzyme system indicates that the specificity of transferase towards tRNA must not be due solely to recognition of the acceptor site at the 3'-terminus of tRNA. It must also involve some other aspect(s) of tRNA structure. The detailed mechanism of this specific recognition has not yet been established, although several proposals have been made (43,79,80).

In order to probe this mechanism, several types of interactions of transferase with polynucleotides have been examined. These studies include nucleoside monophosphate incorporation, substrate binding, and thermal inactivation. The results suggest that the recognition of RNA by transferase is achieved by at least a two-step interaction. Non-specific binding occurs first and is followed by specific complex formation, an event which depends on the specific secondary (and tertiary) structure of tRNA.

Results

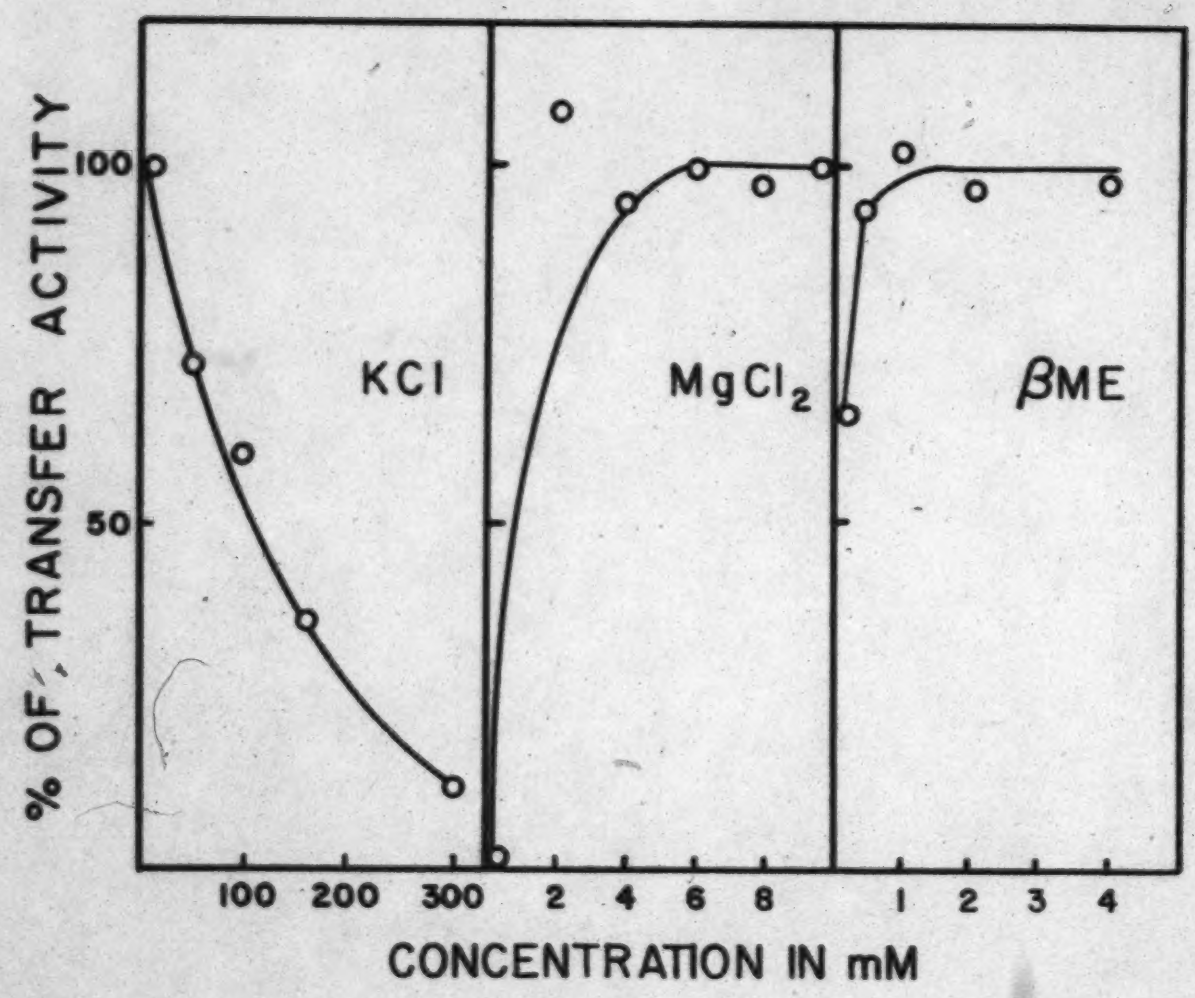
Kinetics of Nucleoside Monophosphate Transfer

Effect of solutes on transfer reaction

During the purification of the enzyme, it was found that a buffer containing 500 mM KCl in 0.1 M Tris-HCl (pH 7.8)--10 mM $MgCl_2$--6 mM βME conferred considerable stability on the enzyme: the protein retained activity over 6 months' storage at -20°C. In view of this stabilizing influence of KCl on the enzyme in storage, the effect of increasing salt concentration on transfer activity was examined. Fig. 1 shows the result. It is clear that KCl inhibits the incorporation reaction. Therefore, in the standard assay system, no KCl was added to the reaction mixtures. However, it should be noted that, in the assay, the KCl concentration was 5-10 mM, due to the addition of the enzyme solution containing 500 mM KCl.

Since the transfer reaction involves nucleoside triphosphates, the optimal ratio of divalent cations (Mg^{+2}) to nucleoside triphosphate was determined. Fig. 1 demonstrates the effect of Mg^{+2} concentration on the initial rate of the transfer reaction. The system has an absolute requirement for Mg^{+2}, but at concentrations higher than 2 mM, the initial rate remains essentially constant. Thus, it was decided to use 5 or 10 mM $MgCl_2$ in the standard assay mixture to ensure that the reaction can proceed even if the experimental design requires changes in the amount of nucleoside triphosphate (NTP). It was found that Mn^{+2} could not be substituted for Mg^{+2}, because the saturation concantration of Mn^{+2} at pH 9 is only 0.4 mM, a level probably insufficient to neutralize the negative charge on the NTP's. (Excess

Figure 1: Effect of reaction components on initial rate of tRNA 3'-terminal nucleotidyl transferase. Assays were performed as described in Materials and Methods. A 20 µg sample of B fraction enzyme and 157 µg of tRNA-pXpC (prepared by the periodate method) were used for the experiments on $MgCl_2$ and βME, while 6 µg of DC fraction (freed of KCl) and 83 µg of s.v.tRNA were used in the experiment on KCl. With B fraction enzyme and tRNA-pXpC, the 100% level of CMP incorporation (initial rate) was normally 210 c.p.m./min/µg protein. For DC fraction enzyme and s.v.tRNA, the 100% level was normally 10,000 c.p.m./min/µg protein.


% OF TRANSFER ACTIVITY
100
50
KCl
MgCl2
βME
100 200 300
2 4 6 8
1 2 3 4
CONCENTRATION IN mM

Mn^{+2} precipitated as manganese hydroxide in the assay mixture). The addition of βME, which is routinely included in storage buffers, was not so essential to the reaction (Fig. 1), but it was included in the standard assay to produce a concentration of 1 mM. The pH effect was also examined. From Fig. 2, it was found that pH 9--9.25 is optimal.

Specificity of transferase for the 3'-terminus of tRNA

It was essential in this series of experiments to have tRNA with an intact 3'-terminus, which would permit preparation of tRNA free of the 3'-terminal adenine and cytosine residues. With these homogeneous species, it would then be possible to examine some aspects of the specificity of transferase activity. A stock of tRNA was found to be satisfactory; this tRNA stock accepts only negligible amounts of nucleoside monophosphates (Fig. 3). After removal of the first residue by a single cycle of treatment with periodate (see Methods section), adenine was released and the tRNA-pXpCpC obtained specifically accepts AMP (Fig. 3). The ATP saturation experiments (Fig. 4) show that there is one AMP residue incorporated for each tRNA-pXpCpC molecule. The results also demonstrate that there is no significant difference between one step periodate-amine cleavage and the more lengthy two-step process. In the second cycle of treatment, cytosine (and some adenine) was released to yield tRNA-pXpC. This form of tRNA accepts only CMP (Fig. 3). Thus tRNA-pXpC does not accept AMP nor does tRNA-pXpCpC accept CMP. These results show that the starting material (tRNA) was intact, and also that the transferase reaction by purified enzyme is specific.

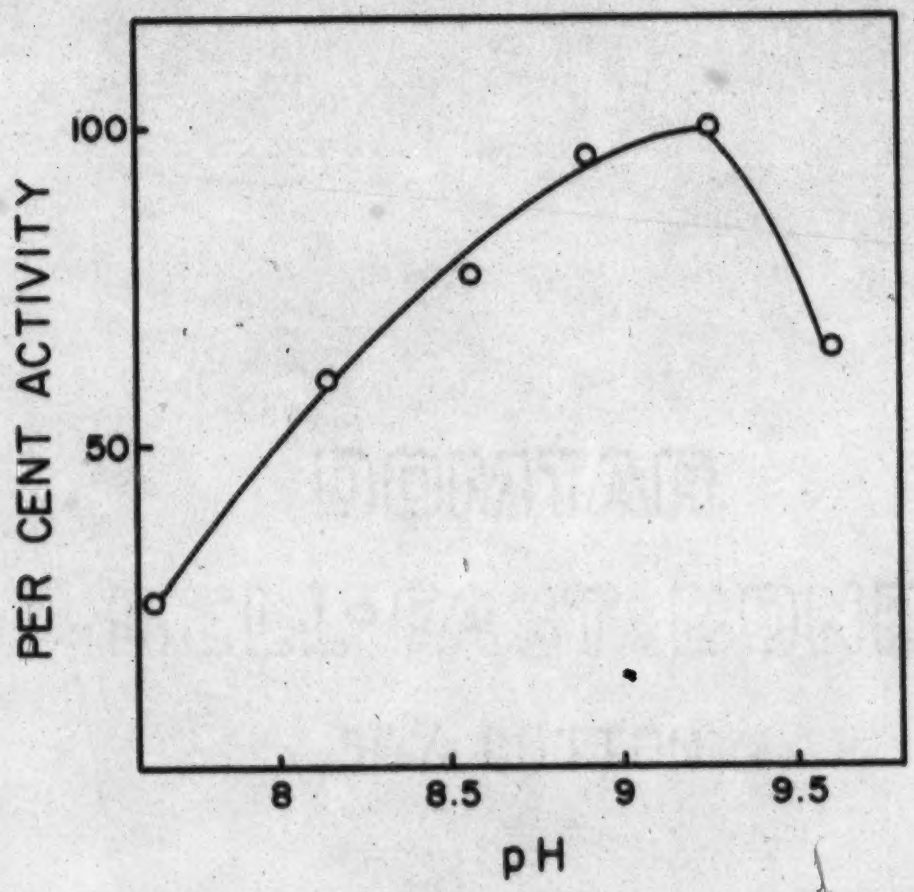


Figura 2: pH profile of the transferase reaction. A 20 µg sample of B fraction enzyme and 157 µg of tRNA-pXpC were incubated at 37°C in the CMP-incorporating system, using Tris-HCl buffers with pH from 7.6 to 9.6. Initial rates were determined as described in Materials and Methods.

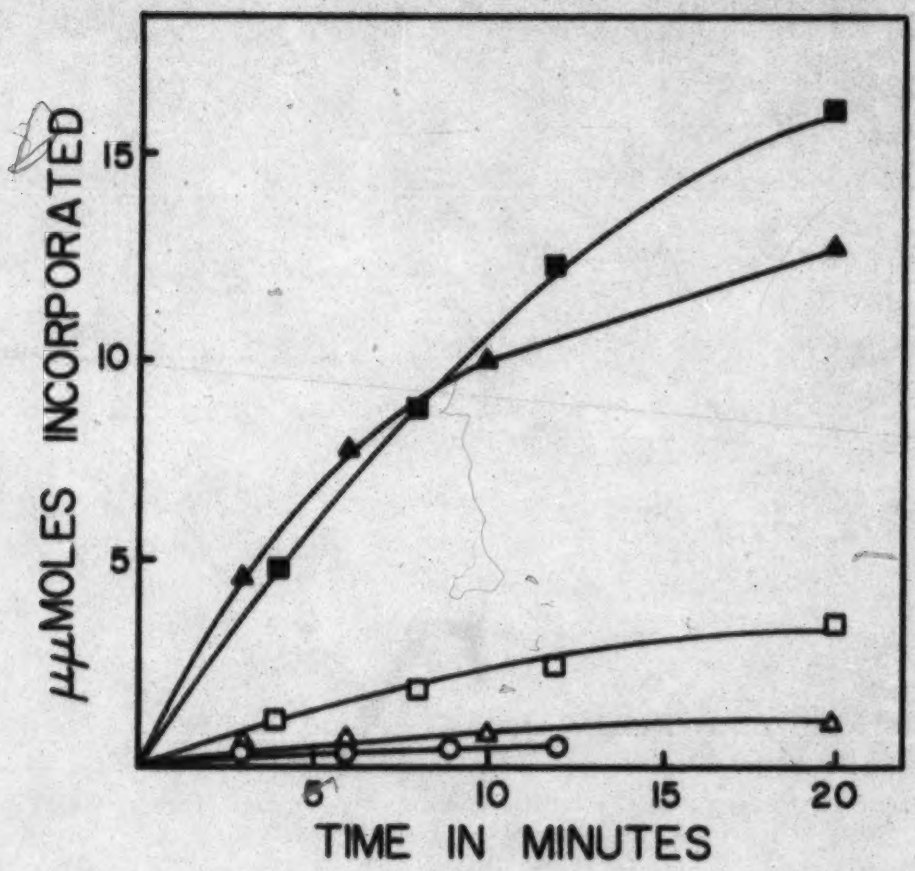
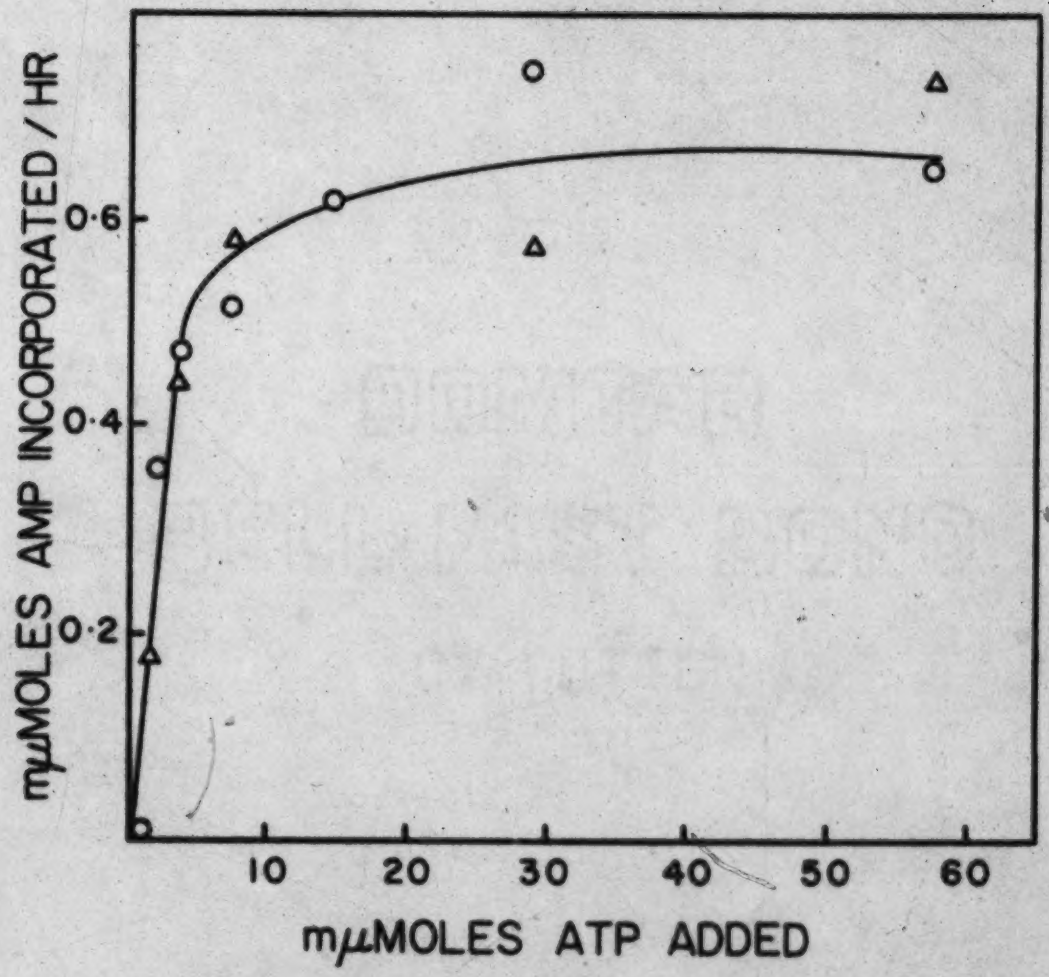


Figure 3: Specificity of transferase for the 3'-terminus of tRNA. Assays were performed as given in Materials and Methods, using 94.5 μg tRNA-pXpCpC and 2.94 μg DC fraction enzyme in the AMP-(■) and CMP-(□) incorporating systems: 130 μg tRNA-pXpC and 2.94 μg DC fraction transferase in the CMP-(▲) and AMP-(△) incorporating systems; and 159 μg untreated tRNA-pXpCpCpA and 6 μg DC fraction enzyme in the AMP- or CMP-incorporating systems (○).

Figure 4: AMP saturation of tRNA-pXpCpC. Assays were performed as outlined in Materials and Methods, using 0.66 mμmoles of tRNA-pXpCpC per 0.09 ml reaction mixture. Total reaction volume was 0.5 ml. The amount of incorporation at 60 min in the presence of increasing amounts of ATP was monitored. Results are plotted in units of 0.09 ml reaction mixture. (○) and (△) are duplicates except that the tRNA-pXpCpC for (○) was prepared by one-step periodate-amine cleavage, as given in Materials and Methods, while that for (△) was prepared by a two-step procedure involving first periodate oxidation, then isolation of the RNA by precipitation, after which amine cleavage is done.


mμMOLES AMP INCORPORATED / HR
0·2
0·4
0·6
10
20
30
40
50
60
mμMOLES ATP ADDED

Since the periodate treatment of tRNA is time-consuming, snake venom phosphodiesterase treated tRNA was prepared as described in the Methods section. When prepared as specified, snake venom phosphodiesterase treated tRNA (s.v.tRNA) accepted only CMP, not AMP. This fact suggests that s.v.tRNA can be used for general assays.

There was no detectable transfer of CMP or AMP to other types of RNA's, including R17 RNA-pC, poly-A, poly-C, and poly-AC. This type of specificity has been demonstrated for the rat liver enzyme by the study of AMP acceptance and of pyrophosphate exchange (28). The present result confirms this finding. However, it was found that these other polynucleotides could inhibit the reaction of transferase with tRNA. Fig. 5 shows some inhibition profiles. For R17 RNA-pC, 50% inhibition occurs at 54 µg (when its concentration equals that of tRNA-pXpC on a weight, rather than a molar basis). The molecular weight of R17 RNA is about 10^6 daltons, tRNA about 25,000 daltons. R17 RNA-pCpCpA was even more effective in inhibiting this reaction. Poly-A and poly-U on the other hand, are much less efficient inhibitors.

Requirement for the secondary structure of tRNA

The above experiments did not show whether the incorporation of nucleoside monophosphates, observed as radioactivity detected in the cold acid precipitates, is due solely to the acceptor activity of the tRNA molecule. In order to test this, the following experiment was performed. One ml of a 10 mg/ml solution of s.v.tRNA was dialyzed against deionized water for 12 hr. This treatment did not effect acceptor activity of s.v.tRNA and 88% of the A_{260} units were recoverable as a CTA precipitate. Heating this dialyzed tRNA at 95°C for 10 min

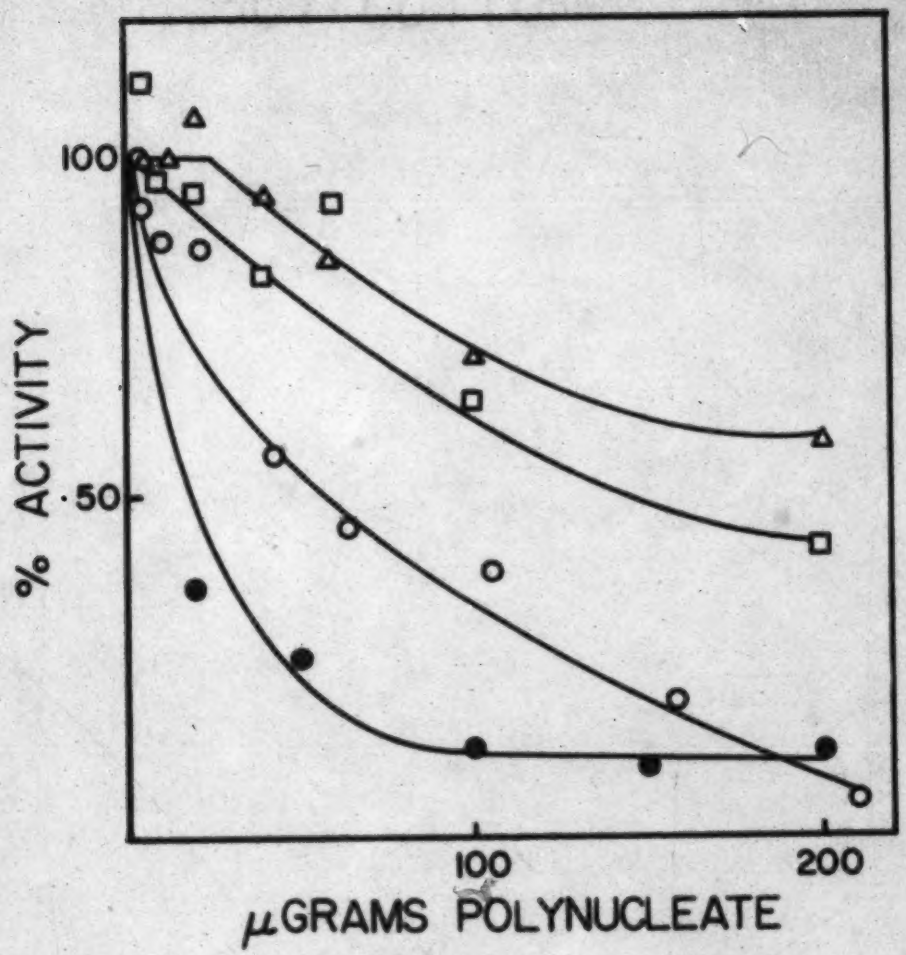


Figure 5: Inhibition of the transferase reaction with tRNA-pXpC by R17 RNA-pC, R17 RNA-pCpCpA, poly-U and poly-A. DC fraction transferase was dialyzed overnight against 0.1 M Tris-HCl (pH 9)--10 mM $MgCl_2$--1 mM βME in order to remove KCl (see text). A 55 μg sample of tRNA-pXpC and varying amounts of R17 RNA-pC (○), R17 RNA-pCpCpA (●), poly-A (□), or poly-U (△) were then incubated in the standard system for incorporating CMP into tRNA-pXpC. Per cent activity was determined from initial rates. The 100% level in this experiment was 10,000 c.p.m./min/unit dialyzed enzyme (approximately 1 μg).

resulted in loss of acceptor activity (Fig. 6) although 89% of the A_{260} units were recoverable as a CTA precipitate. A control experiment showed that the treatment caused no loss of radioactivity via tritium exchange. Hence, it can be concluded that the acceptor molecule was non-dialyzable and that the secondary structure of this molecule is of considerable importance in the transferase reaction (a similar result was obtained by Lindahl et al (56) for AMP incorporation into renaturable leucyl-tRNA).

Interaction of Transferase with RNA's

In order to obtain some insight into the mechanism of the specific reaction of transferase, the initial stage of the overall reaction, which is the interaction of the enzyme with RNA, was studied using a membrane filtration method, as described in the Methods section.

Enzyme retention on Millipore filters

In order to assess transferase retention on the filters, enzyme was dissolved in several different buffers, filtered, and washed with the corresponding buffer. The filtrate and wash solutions were pooled and assayed for transferase activity. Also, each filter disc was cut into pieces and assayed. It was found that the concentrations of KCl, $MgCl_2$ and βME have no effect on enzyme retention, while increasing pH reduces retention. At pH 5.6, all detectable activity was retained on the filter, but at pH 6.4, retention decreased to 1/10th. At pH 7.5 or higher, the filter retained essentially no activity. Potassium phosphate buffer concentration also appeared to be important, in that at concentrations below 15 mM, there was loss of enzyme from the filters. In accord with these findings, the binding

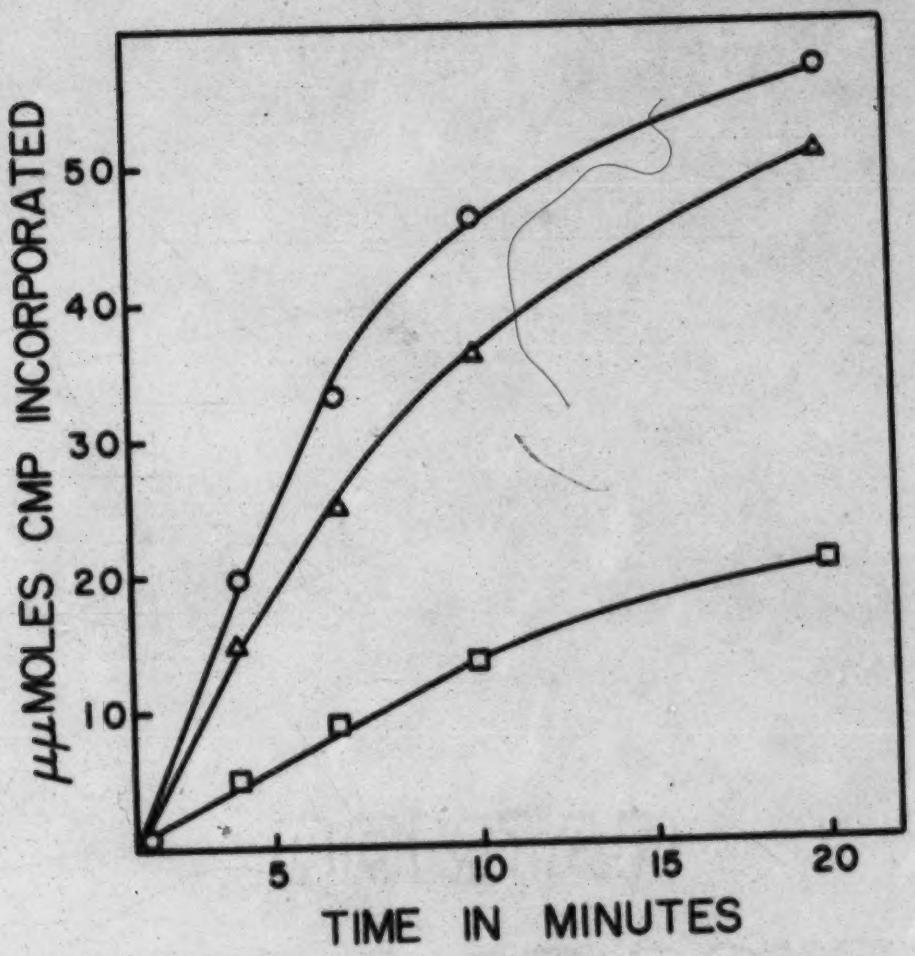


Figure 6: CMP acceptance of denatured s.v.tRNA. s.v.tRNA was dialyzed 24 hr against deionized, distilled water. An aliquot of this was heated in a boiling water bath (95°C) for 10 min and quick cooled on ice. Incorporation of CMP into each tRNA species was then determined: 166 μg native s.v.tRNA (○), 69 μg dialyzed s.v.tRNA (△), 69 μg dialyzed and heated s.v.tRNA (□).

study using Millipore filters was performed at pH 5.6 (20 mM potassium phosphate buffer).

It was essential to use RNase-free transferase (DC fraction) throughout the following studies under the conditions defined above. Contamination by ribonuclease would result in anomalous results for the binding of tritiated or [^{14}C]-labelled RNA to enzyme, caused by hydrolysis and loss of radioactivity.

Solute concentration effects on complex formation

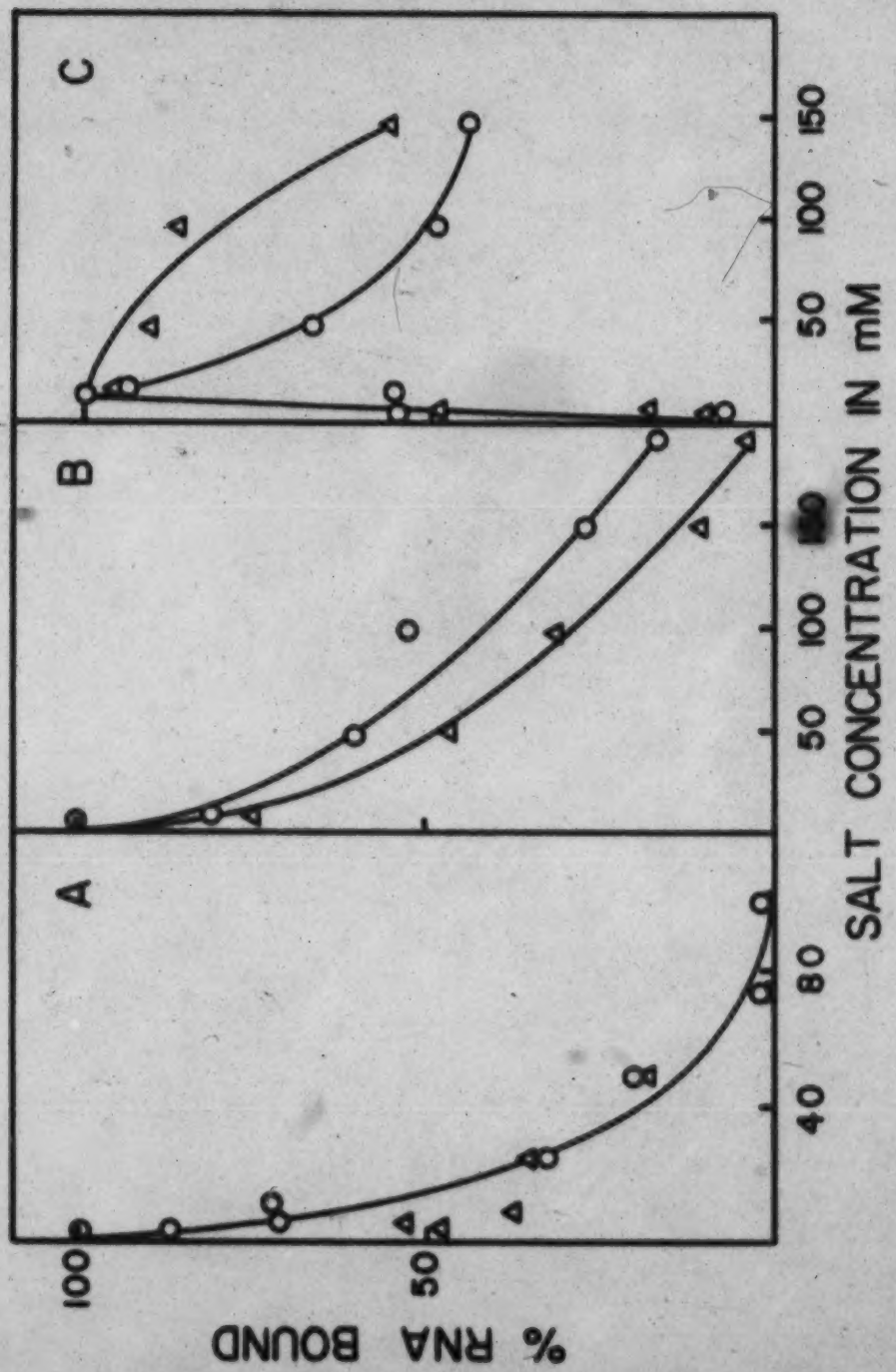
Fig. 7 shows the effect of [Mg^{+2}] on binding of [^{3}H]CMP-tRNA and [^{14}C]s.v.R17 RNA to transferase. The results demonstrate that at low $MgCl_2$ concentrations, both RNA species can bind to the enzyme, and binding falls off in a similar manner with increasing [Mg^{+2}]. Since Mg^{+2} concentration has no effect on enzyme retention, the conclusion seems valid.

Fig. 7 also shows the effect of KCl concentration on the binding of [^{14}C]s.v.R17 RNA and of [^{3}H]CMP-tRNA to the enzyme. It is again observed that increasing concentration of KCl inhibits complex formation.

Fig. 7 also demonstrates the effect of potassium phosphate concentration on the complex formation. Since enzyme retention is reduced by low phosphate concentration, the early part of the curve is not likely significant with regard to binding. Optimal binding occurs, in both cases, at about 20 mM potassium phosphate.

Binding of both RNA species was unaffected by changes in βMe concentration over the range from zero to 10 mM.

Figure 7: Effect of salts on RNA binding to nitrocellulose-bound transferase. A 13.3 µg sample of transferase was incubated with either 42 µg [^{3}H]CMP-tRNA (○) or with 8.6 µg [^{14}C]s.v.R17 RNA (△) in the appropriate buffer and filtered through Millipore discs. The effects of $MgCl_2$ concentration (A), KCl concentration (B), and potassium phosphate concentration (C) were monitored. The 100% level of binding for tRNA in this experiment was 1.23 µg/13.3 µg enzyme, and for R17 it was 2.84 µg/13.3 µg enzyme.


% RNA BOUND
100
50
A
B
C
40
80
50
100
150
SALT CONCENTRATION IN mM

The above three experiments clearly demonstrate the presence of complex formation between enzyme and tRNA and R17 RNA, under appropriate conditions: 20 mM potassium phosphate (pH 5.6)--5 mM βME in the absence of $MgCl_2$ and KCl, in the cold.

Stoichiometry of binding

Figure 8 shows the saturation profiles for binding of RNA to a fixed amount of the enzyme (6 µg). tRNA and R17 RNA show identical saturation profiles with saturation being reached when about 5 µg of either type of RNA are added. Thus, saturation levels of the two systems compare on a weight basis, but not on a molar basis (R17 RNA is about 40 times as large as tRNA). This fact suggests that complex formation under the conditions described above is due to non-specific RNA base-protein interaction. If it were due to interaction with a specific site of RNA, saturation levels of the two RNA species should show molar equivalence.

The above speculation can perhaps be substantiated by studying complex formation between the enzyme and heat denatured tRNA, a species which has impaired acceptor activity in the transferase reaction (Fig. 6). The denatured [^{3}H]CMP-tRNA was examined for binding capacity. From Table 1, it is clear, however, that denaturation of tRNA reduces (but does not abolish) binding capacity. The reduction factor in transfer activity is 3, while in binding activity it is 2.4. Thus, it is apparent that configurational change in the tRNA molecule alters both transfer and binding activity.

In order to obtain more insight into the nature of binding under the present experimental conditions, binding of synthetic

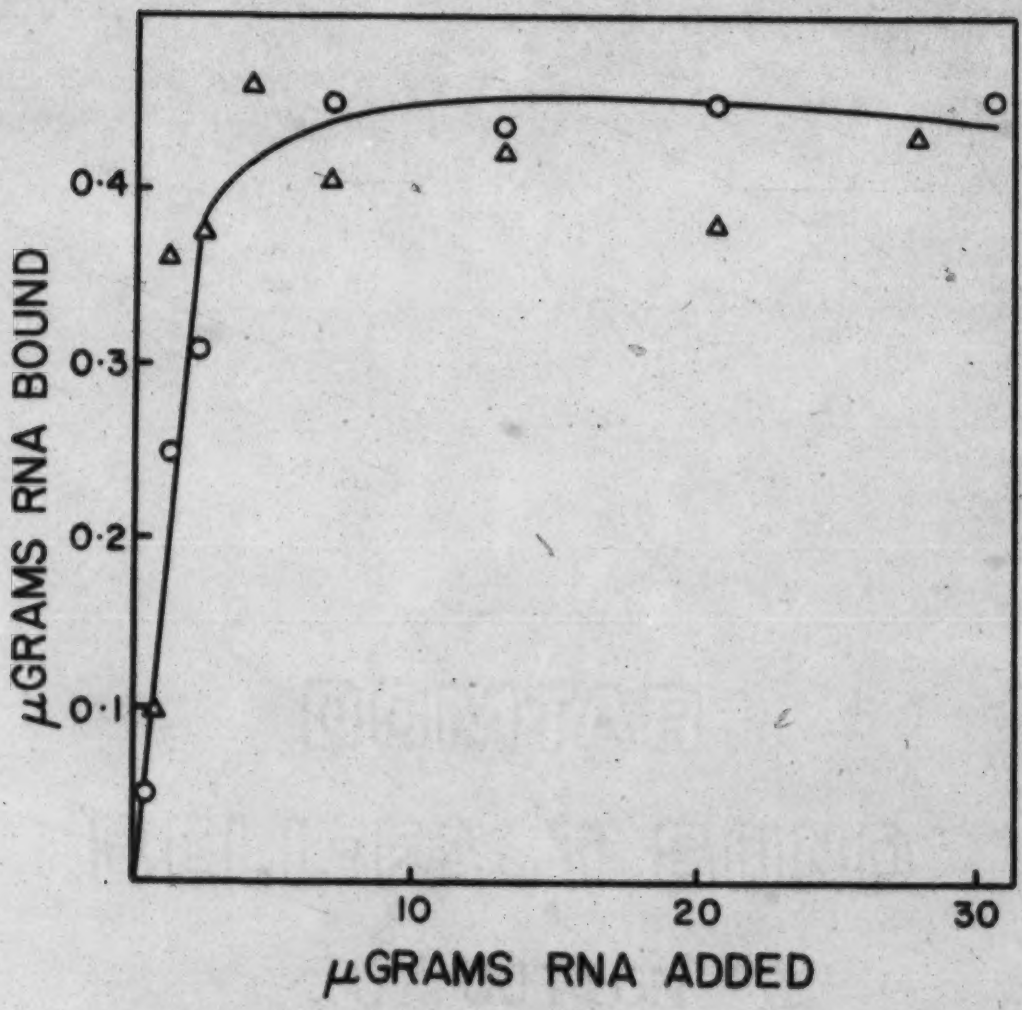


Figure 8: Binding of tRNA and R17 RNA to transferase. Binding experiments were performed as described in the text. A 6 μg sample of transferase was incubated with varying amounts of [^{3}H]CMP-tRNA (O) or [^{14}C]s.v.R17 RNA (Δ), and the complex was obtained on Millipore filters. Radioactivity retained on the filters was converted to micrograms bound RNA.

TABLE 1. Binding of Denatured [^{3}H]s.v.tRNA to Transferase

	Input		Retained on filters	
s.v.tRNA-[^{3}H]CMP	µg	c.p.m.	c.p.m.	% retained
Native	19	76,000	2687	3.54
Dialyzed	12	59,000	2120	3.55
Dialyzed and heated	12	59,000	905	1.51

polynucleates was examined. The molecular weights of these synthetic RNA's varied somewhat, but averaged approximately 200,000 daltons. It is very clear from Table 2 that all synthetic polynucleates tested can bind to the transferase. In this table, a very rough measure of relative association tendency is given. The assumption is that an equilibrium exists as:

$$X + \text{Enzyme} \rightleftharpoons Z,$$

where X is polynucleotide phosphorus, and Z the enzyme-polynucleotide complex, whose exact nature is unspecified. The association tendency can then be expressed as:

$$K = \frac{Z}{X} \cdot \frac{1}{\text{Enzyme}} .$$

At constant enzyme concentration, a relative association tendency can be written as:

$$\varepsilon = \frac{Z}{X} \text{ or } \frac{\text{c.p.m. bound}}{\text{c.p.m. unbound}} .$$

In Table 2, ε is given for 10 µg protein. The DC fraction, which is RNase-free, binds more of each polynucleate than does the less pure B fraction. There is particular enhancement of the poly-U-specific binding.

The degree of base stacking seems to have bearing on the extent of binding. It is known from study of the interaction of heterocycles that purines self-associate by stacking to a greater extent than do pyrimidines (81). The table shows that purine polymers (poly-A and poly-G) are less able to bind to the more purified enzyme than are the pyrimidine polymers (poly-U and poly-C). Thus, greater

TABLE 2. Binding of Synthetic Polynucleotides to Transferase

Synthetic polynucleotide	ε/10 µg Protein	
	Fraction B enzyme	Fraction DC enzyme
^{3}H poly-U	1.31	27.1
^{3}H poly-C	15.4	16.9
^{3}H poly-G	9.36	12.3
^{3}H poly-A	6.40	9.95
^{3}H poly-AU	18.1	13.3
^{3}H poly-CU	19.8	25.9

stacking appears to correlate with less binding capacity. This may explain the effect of heat denaturation of tRNA on binding: heating and quick-cooling destroys the ordered secondary hydrogen-bonded structure of tRNA, and may result in a greater amount of stacking-type interaction. This would then reduce the binding capacity of the tRNA.

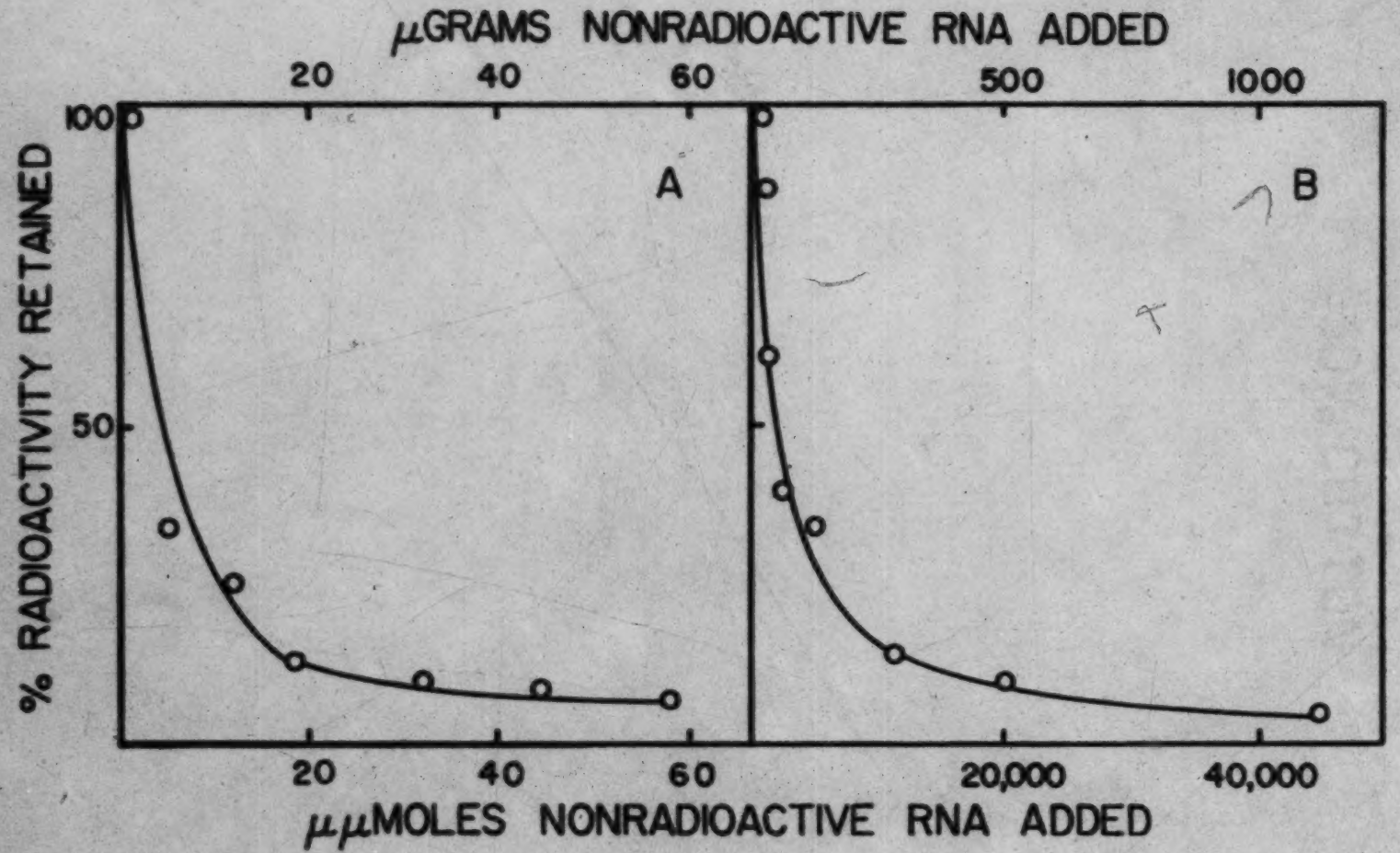
Competition between RNA's for binding

If R17 RNA and tRNA are binding to the same enzyme then they should compete for the common binding site(s). From Fig. 9, it is clear that both RNA species can be dissociated from a complex with the enzyme by the other species. The amount of unlabelled RNA needed to reduce the amount of bound labelled RNA to 50% of the control is designated the C-50 point. The C-50 point for competition with labelled tRNA is obtained at a 0.16 molar excess of R17 RNA (25.1 μmoles of tRNA compete with 4.1 μmoles R17 RNA), but at a microgram excess of 6.4. On the other hand, the same values for competition with labelled R17 RNA are 396 molar excess, but only 9.9 μg excess of tRNA. The C-50 values on a weight basis (6.4 and 9.9) are much closer than the values on a molar basis. This observation agrees well with the finding that similar weight quantities of each RNA can bind to the enzyme (see Fig. 8).

Thermal inactivation of transferase

It is now obvious that transferase can interact with at least all the RNA types tested, under the conditions employed. In order to further understand this interaction, the thermal inactivation profiles in the presence and absence of RNA were examined. It was important to

Figure 9: Competition between tRNA and R17 RNA for binding to transferase. A 12.1 µg sample of DC fraction enzyme was incubated in the cold with 25.1 µµmoles (0.63 µg) of [^{3}H]CMP-tRNA and varying amounts of unlabelled s.v.R17 RNA (A). Similarly, the enzyme was incubated with 2.7 µµmoles (2.7 µg) of [^{14}C]s.v.R17 RNA and varying amounts of unlabelled s.v.tRNA (B). Each mixture was filtered through Millipore discs which were dried and counted.

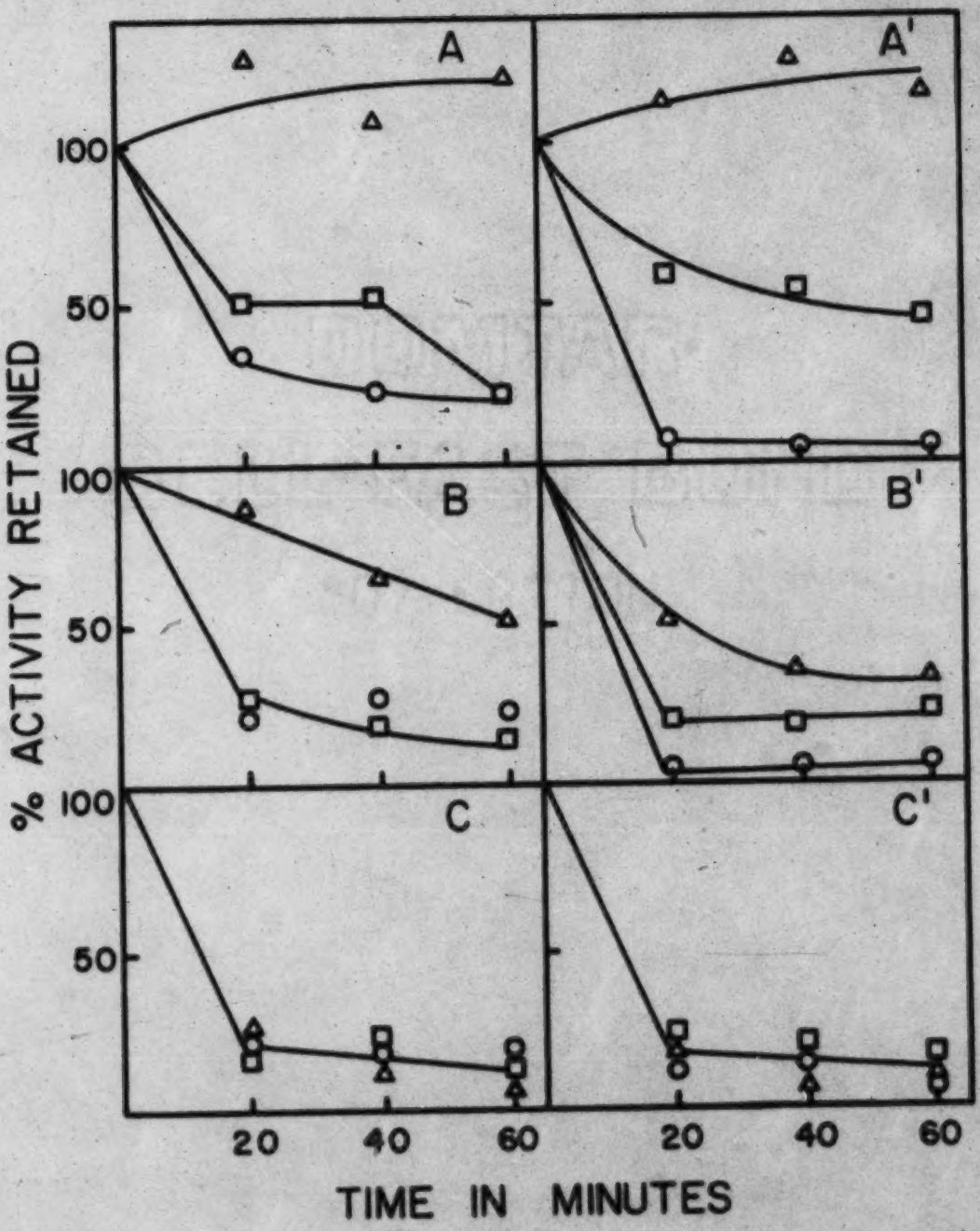

μGRAMS NONRADIOACTIVE RNA ADDED
20
40
60
500
1000
100
50
% RADIOACTIVITY RETAINED
A
B
20
40
60
20,000
40,000
μμMOLES NONRADIOACTIVE RNA ADDED

discover whether the inactivation profiles were affected by pH since the binding study was done at pH 5.6 and the incorporation study at pH 9.0. Differences in enzymic behaviour at the two pH values could cause misinterpretation of the results in the binding study. The question then is whether the specific transfer reaction with tRNA is accompanied by some conformational change in the enzyme, to result in stabilization of the complex, or whether the reaction occurs so that the frequency of transfer is proportional to the frequency of binding of 3'-termini and enzyme (i.e., the longer the RNA chain, the lower the frequency). Thermal inactivation studies may indirectly distinguish these alternatives.

Fig. 10 shows the thermoinactivation of transferase at three temperatures. It becomes clear from this figure that the enzyme can be protected at 50°C, in the presence of tRNA, but not in the presence of R17 RNA. Without added RNA, or in the presence of R17 RNA, enzyme inactivation occurs quite readily at this temperature. At 55°C, there is partial protection of the enzyme by tRNA, and at 60°C, inactivation occurs regardless of the presence or absence of added RNA. An important fact found in this thermal test is that at each temperature, pre-incubation pH does not significantly alter the inactivation profiles. This fact lends support to the validity of the earlier binding study in determining the affinity of enzyme towards RNA's.

Since synthetic polynucleotides, such as poly-A and poly-U can bind to transferase and can inhibit the transferase reaction with tRNA, the effect of these polynucleates on thermal stability of enzyme was examined. Neither protects the enzyme against heat inactivation, as seen in Fig. 11. Thus they behave similarly to R17 RNA in this

Figure 10: Thermal inactivation of transferase in the presence of tRNA and R17 RNA. Heat inactivation studies were done as described in Materials and Methods. Enzyme was dialyzed overnight against the appropriate buffer. In the control experiments (□), 6 µg of DC fraction was incubated at pH 5.6 (A, B, C) or pH 9 (A', B', C') at 50°C (A, A'), 55°C (B, B'), or 60°C (C, C'). Samples containing 133 µg s.v.tRNA (△), and samples containing 125 µg s.v.R17 RNA (○) were treated in the same way. Initial rate of CMP incorporation into s.v.tRNA at 37°C by the enzyme mixtures pre-incubated at the temperatures given was then determined. Results are plotted as per cent activity versus pre-incubation time at the high temperature. (It should be noted that the presence of R17 RNA in the reaction vessel invariably caused a reduction in the 100% value for that set).


A
A'
B
B'
C
C'
100
50
100
50
100
50
% ACTIVITY RETAINED
20
40
60
20
40
60
TIME IN MINUTES

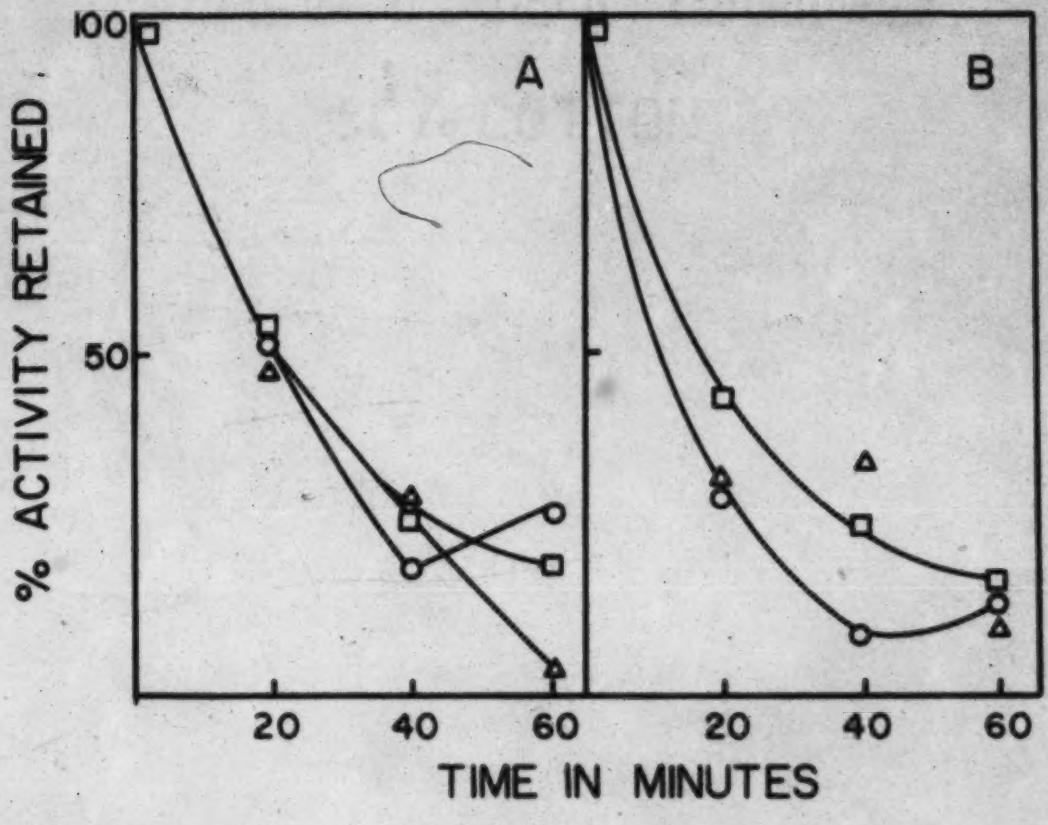


Figure 11: Thermal inactivation of transferase in the presence of poly-A and poly-U. Heat inactivation was performed as outlined in Fig. 10 at pH 5.6 (A) and pH 9 (B), at 55°C. Control without polynucleotide (□) contained about 15 µg DC fraction. Controls and samples containing 50 µg poly-U (△) or 50 µg poly-A (○) were subjected to identical incubations at 55°C for various lengths of time and then enzyme activity assayed at 37°C using 50 µg tRNA-pXpC and CTP. Extent of reaction at 12 min is plotted as per cent activity versus pre-incubation time at 55°C.

system. (During the course of this work, Miller and Phillipps (43) reported thermal inactivation studies, in which they observed protection by tRNA but no effect by synthetic polynucleotides or rRNA or 5S RNA). It is now clear that there is thermostable specific complex formation between transferase and tRNA prior to the transfer reaction.

Discussion

As was pointed out in Chapter I, some *E. coli* transferase preparations do cause such misincorporation reactions as:

$$\text{tRNA-pXpC} + \text{ATP} \longrightarrow \text{tRNA-pXpCpA} \qquad (41),$$

while others do not (43). In each of the references quoted above, enzyme preparations were highly purified, but not completely homogeneous. The type of specificity demonstrated in this thesis agrees with the results of Miller and Phillipps (43): transfer RNA which lacks a terminal AMP (tRNA-pXpCpC) accepts only AMP, while tRNA lacking both a terminal AMP and CMP (tRNA-pXpC) accepts only CMP. s.v.tRNA prepared in the standard manner, as given in Materials and Methods, cannot accept multiple CMP residues (the CTP saturation experiment shows that 78.5% of the tRNA molecules accept one nucleotide). Untreated tRNA is not an acceptor of either AMP or CMP. The differences between these results and those of Best and Novelli (41) may be due to (a) lack of complete homogeneity in either preparation, so that misincorporation may be the result of a non-transferase enzyme; or (b) the possible presence of multiple transferase species: the two laboratories may have isolated different species of transferase which differ with respect to misincorporation. The actual cause

for the above discrepancy is not resolvable at present. However, it must be emphasized again that the present E. coli transferase does display absolute specificity. Thus this enzyme must possess ways of distinguishing tRNA-pXpC and tRNA-pXpCpC and of distinguishing ATP and CTP, under the conditions employed.

It has already been determined that there are separate enzymatic sites for ATP and CTP (34,35). The question thus arises as to the nature of the site or sites involving transfer RNA. The site(s) can distinguish different 3'-terminal sequences, as is demonstrated by the specificity discussed above. It is apparent, however, that the enzyme is not responsive only to the sequence of mononucleotides at the 3'-teminus of RNA. Denaturation of tRNA by dialysis and heat results in a 67% loss of incorporating activity. In addition, neither R17 RNA-pC (with a 3'-terminus similar to tRNA) nor poly-C show incorporation of CMP. These facts imply that there is a recognition site or sites other than the 3'-terminus of tRNA. Incorporation of mononucleotides into non-tRNA substrates has been observed in mammalian systems (38,39). However, these reactions occur in the presence of Mn^{+2} instead of Mg^{+2} or at high enzyme concentrations. Under normal conditions, only tRNA is the nucleic acid substrate.

Binding experiments were undertaken to help explain some of the questions about the recognition mechanism. A study of the binding of transferase to yeast tRNA, MS2 RNA, and cRNA has been done by Honda (59), using a sucrose gradient centrifugation method to detect complex formation. It was felt that a gentler means of assaying binding should be used because it was possible that the complexes

can be disrupted under these conditions (69). Therefore, the interaction of various types of RNA with the transferase was studied using a milder Millipore filtration method.

Denaturation of tRNA by dialysis and heating affects the binding activity in the same way as it affects incorporation activity: native and dialyzed tRNA bind identically, while heated dialyzed tRNA shows a 57% loss of binding activity. Thus, it seems that denaturation is sufficient to result in considerable loss of binding ability, leading to loss of incorporating activity.

The foregoing indicates one effect of RNA structure on the association of transferase and RNA. The binding of synthetic RNA's may throw further light on this problem. As seen in Table 2, purified transferase has different affinities for different synthetic polynucleates: poly-U>poly-C>poly-G>poly-A. Poly-AU binds with an affinity which is greater than that of poly-A but less than poly-U, and poly-CU binds better than poly-C but not so well as poly-U.

It has been established that self-association of heterocyclic compounds in aqueous solution is predominantly due to base stacking, which in turn is dependent on the degree of π-electron conjugation (81). Cursory examination of the structures of the nucleosides (A, U, G, and C) shows that the order of size of π-electron systems is $U \leq C < G \leq A$. This is the reverse of the order obtained for the binding capacities of the polynucleotides. These results are strongly suggestive that binding capacity is inversely related to extent of base stacking. It seems possible that a freely rotating state of RNA (which is hindered by base stacking) is essential to non-specific interaction with protein (82). This is consistent with the fact that

denatured tRNA, which is expected to have more base stacking than native tRNA, has less binding capacity.

The studies by Honda gave different results from those presented here, in that he observed no binding to MS2 RNA or rRNA, while binding to R17 RNA and to synthetic polynucleotides has been observed in this lab. This difference may be the result of the fact that the centrifugation method may be too harsh, especially for complexes with high molecular weight RNA. In passing, it should be noted that Morris and Herbert were unable to demonstrate binding of their yeast enzyme to poly-U or poly-C, using a Millipore method (60). Unfortunately, their Millipore method differed significantly from the one reported here. Binding experiments were done at pH 6.5, while in the present study, pH was 5.6. The study presented in this chapter, indicated that the E. coli enzyme is not retained well on Millipore filters at pH 6.5. It is hard to judge what effect this would have. One conclusion may be that the yeast and E. coli enzymes are different enough to account for the lack of agreement between the two laboratories.

The present studies have demonstrated that both tRNA and R17 RNA can bind to transferase. Competition experiments show that one RNA species can inhibit complex formation between the enzyme and the other polynucleate. The fact that the C-50 values compare on a weight basis and the fact that the saturation profiles of tRNA and R17 RNA binding with enzyme are identical on a weight basis suggest that the binding is the result of non-specific nucleotide-protein interaction, rather than a specific nucleic acid-protein interaction. At this point, it may be useful to comment that although Morris and Herbert cannot demonstrate binding of poly-U or poly-C to yeast transferase,

they find that these homopolymers are able to inhibit the binding of transfer RNA (60). This suggests that there is some artefactual reason for their lacking of binding.

A test of the validity of the binding study is to determine whether the non-accepting RNA species affect the transfer reaction with tRNA. If the non-accepting species bind to the enzyme, and if this binding is at or close to the catalytic site, then they may inhibit the transfer reaction with tRNA. In fact, although R17 RNA and the synthetic polynucleates tested could not accept nucleoside monophosphates in the transfer reaction, they were able to inhibit the incorporation of CMP into tRNA-pXpC. It should be noted at this point that the effectiveness of inhibition varied considerably depending on the conditions of the experiment. In the presence of high KCl concentrations, such as that used to stabilize the enzyme solution (HSB), inhibition of the transfer reaction by non-accepting nucleic acids is abolished. Miller and Philipps observed no inhibition of the transfer reaction by synthetic polynucleotides in their E. coli system (43). They used 2.5 mg/ml bovine serum albumin in their enzyme solution as a stabilizer and do not mention removing this prior to any of their studies. This may be the reason for their negative result.

Differences in the extent of inhibition by the several RNA's tested are noteworthy. The natural RNA inhibits much more efficiently than the synthetic polynucleates. Poly-A (and poly-C) inhibit slightly more effectively than poly-U. If the inhibition were due solely to the non-specific type of interaction found in the binding studies, then poly-U should be a more potent inhibitor than poly-A and poly-C. Likewise, there should be little differences between the

inhibition of untreated R17 RNA and that of R17 RNA-pXpC. Yet, there is a consistent, marked difference: R17 RNA inhibits at a lower concentration than R17 RNA-pXpC. The explanation may lie in the possibility of closer association of the 3'-terminus of the natural polynucleate with the transfer site on the enzyme. It is known that there are separate sites on the enzyme for the binding of the substrates ATP and CTP. Poly-A and poly-C may be capable of interaction at these sites. This would have the effect of increasing their efficiency as inhibitors. Similarly, R17 RNA with an intact 3'-terminus may be capable of interaction at these sites because of the presence of the terminal -pCpCpA.

The non-accepting RNA's, then, do complex with transferase and, to varying extents, inhibit its reaction with tRNA-pXpC. It is useful to consider whether tRNA, R17 RNA, and/or the synthetic polynucleates differ with respect to heat inactivation of the enzyme. This would determine whether these RNA's, which behave similarly in initial binding, are able to proceed beyond that stage in the process of the reaction. The thermal inactivation studies reveal that, as expected, tRNA protects the enzyme from thermal inactivation at 50°C. This protection is less efficient at 55°C and fails entirely at 60°C. Presumably the kinetic energy of the complex is too great at 60°C to maintain the complex structure. Transfer RNA departs from the complex, leaving the enzyme "naked" so that it undergoes thermal inactivation at the same rate as the control with no RNA present. Under conditions in which tRNA protects the enzyme from heat inactivation, no RNA's of other types protect transferase. Thus it is clear that tRNA complexes with transferase in a manner different from the other

polynucleates, possibly resulting in structural change in the enzyme upon binding.

It is now evident that there is association possible between R17 RNA (as well as other polynucleates) and transferase. It is of a non-specific nature and does not seem to directly involve the site of reaction on the enzyme, since these species do not accept nucleoside monophosphates and they cannot protect the enzyme from thermal inactivation. This suggests the existence of another type of interaction, a more specific one, which requires the special structure of tRNA. There may be a conformational change involved or simply a spatial arrangement necessary to permit close association of the 3'-terminus with the active center and the nucleoside triphosphate substrate.

CHAPTER 4

EFFECT OF ACRIDINES ON THE TRANSFERASE INCORPORATION REACTION

Introduction

After the basic characteristics of the E. coli tRNA 3'-terminal nucleotidyl transferase had been defined, it was decided to probe in turn the components of this system. Through individual investigations of transfer RNA and enzyme, it was hoped that further information could be obtained about the way in which these two important components interact. The search first was for a substance which would alter the behaviour of tRNA in the enzymic reaction without producing an effect on the enzyme. If the alteration in tRNA behaviour can be correlated with structure, some insight may be forthcoming about the association between tRNA and enzyme.

A convenient tool with which to pursue this aim is the acridine dyes. It has been known for some time that acridine orange and its analogs, some of which have been used as antiseptics, are potent inhibitors of many reactions involving nucleic acids (83,84). These dye molecules are fused heterocyclic aromatics which contain either a quaternary ammonium group or an amine group that is positively charged at pH values lower than 10. Thus the molecules are capable of at least two types of interactions: (I) hydrophobic interactions involving the aromatic ring system and (II) hydrophilic interactions involving the charged part of the molecule. It is generally agreed that there are indeed two types of interaction between

the acridines and nucleic acid. DNA, which is basically a hydrogen-bonded double helical structure, associates with these dye molecules by intercalation, at low dye to DNA nucleotide ratios (85). At high dye to DNA ratios, the dyes interact via the formation of a dye pseudo-polymer along the DNA. On the other hand, RNA does not have much double helix but maintains a more extended conformation, and it is capable of the second type of interaction (86). It is a non-specific association in which the charges are of paramount importance and which requires a high dye to RNA nucleotide ratio. The dye molecules self-stack with their positive charges aligned toward the negative phosphate charges of the polynucleate.

It may be useful at this point to indicate some differences between intercalation and base stacking. Base stacking is an unstructured process involving hydrophobic (π-electron type) interaction between aromatic molecules. There may be only two aromatic moieties involved, or many more. Intercalation derives its energy of stabilization from the same source (π-electron sharing) but differs from base stacking in that there is a structural requirement for insertion of one small aromatic residue between too nucleotide moieties that are in a helical structure. Thus fewer small molecules can associate with polynucleates via intercalation than via base stacking.

Transfer RNA is believed to possess a unique conformation due to extensive intramolecular hydrogen bonding, which results in a clover-leaf structure. Thus tRNA molecules have two structural elements: double helical regions separated by single stranded sections. Due to these characteristics of tRNA, it can be expected, and in fact has been found, that tRNA binds dyes by intercalation under certain

conditions (87-90). The question thus arises as to what causes the inhibition of tRNA functions by acridine dyes: is it due to intercalation of dye molecules in double helical regions of tRNA, or is it due to ionic interaction with the negative phosphate charges of the polynucleates?

It was hoped that an understanding of the nature of the inhibition by the acridine dyes would indirectly shed some light on the mechanism of tRNA recognition by the enzyme. The transferase system is well fitted to assess the effect of the dyes. The pH optimum for the reaction is 9, but at pH 8 and 10, activity can be measured that is half the maximal rate. The dissociation constant for the acridine amines is 9.6 - 10.5 (91). Therefore, by manipulating pH, it is possible to determine the effect of the acridine charge properties on the inhibition of the transferase reaction. Other nucleotidyl transferase systems whose pH optima range from 7 to 8 do not permit this type of analysis (26,27).

Another useful aspect of this system is that there are available many chemical analogs of acridine which permit one to assess the effects of different substitutions, for example bulky substituents at different ring positions.

The third and final advantage in using the acridines is that they are fluorescent molecules. Fluorescence is very sensitive to molecular environment so that it can be used as a tool to determine the nature of the physical association between tRNA and acridines.

The results presented below indicate that acridines do inhibit this reaction, but at higher concentrations than are expected for intercalation. Inhibition is partially dependent on the presence

of a positive charge on the dye molecule. Relative inhibitory power varies depending on the type of substitution on the fused ring system. Fluorescence studies indicate that interaction between tRNA and the dyes (in the inhibitory concentration range) involves stacking between the RNA bases and the dye molecules. Moreover, the first stage of the reaction (binding) is the one that is sensitive to these dyes. This prevents catalysis. The results do not support the hypothesis of inhibition by intercalation, nor do they support the concept of pseudopolymer formation. Rather they suggest a two-stage interaction: the first is ionically mediated. The positive charge of the dye interacts with the negative phosphate charges on the polynucleate. Then the aromatic portion of the dye is brought into stacking interaction with the aromatic bases of the RNA.

Results

Inhibition by Acridine Orange and Ethidium Bromide

Inhibition of the transferase reaction by these dyes was first examined in the CMP-incorporating system at pH 9. The data are presented in terms of dye concentration vs. initial rate of CMP incorporation, taking the rate of the reaction in the absence of dye as 100%. Fig. 12 demonstrates that both acridine orange and ethidium bromide inhibit this reaction. Acridine orange reduces the reaction by a half at about 400 μM, ethidium bromide at about 200 μM. At this point, it should be noted in passing that the concentration of tRNA nucleotides in the reactions is about 600 μM.

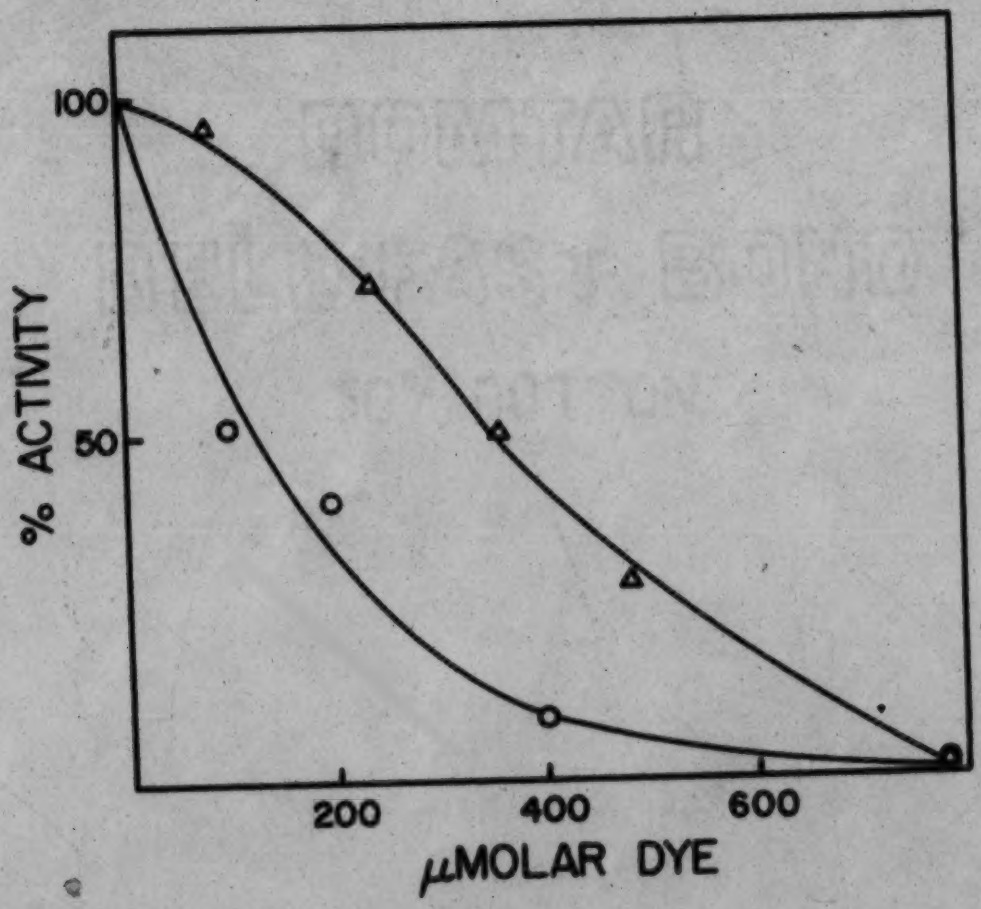


Figure 12: Inhibition of transferase by acridine orange and ethidium bromide. Assays were performed as given in Materials and Methods, using 92.8 μg s.v.tRNA and 13.4 μg DC fraction. The initial rate of CMP incorporation in the absence of added dye was taken as 100%. Activity was examined in the presence of varying amounts of acridine orange (△) and ethidium bromide (○). 100% activity in these experiments was about 4200 c.p.m./min/μg protein.

In order to investigate the expectation that inhibition was due to interaction with tRNA, a pre-incubation study was undertaken. These results are shown in Table 3. When a given concentration of acridine orange (AO) is pre-incubated on ice with s.v.tRNA, 83% inhibition of the subsequent incorporation assay at 37°C is observed; when AO is pre-incubated with enzyme alone, only 71% inhibition occurs; while pre-incubation with both enzyme and s.v.tRNA results in 89% inhibition. These results do not distinguish clearly whether the sites of inhibition were tRNA or enzyme. In order to provide more definitive data, a concentration study was done. If changing the concentration of a reacting substance alters the amount of inhibitor required to produce an inhibition profile, that is good evidence that the substance in question is the site of the inhibitory action. The only reaction component which has this effect is tRNA. Fig. 13 shows the acridine orange inhibition curves for AMP and CMP incorporation into tRNA-pXpCpC and tRNA-pXpC respectively, at two different tRNA concentrations. For this dye, a three-fold increase in tRNA concentration causes about a three-fold increase in the amount of acridine orange needed to produce inhibition. Fig. 14 shows a similar result for ethidium bromide inhibition. Once again, increasing the tRNA concentration causes an increase in the amount of dye needed to inhibit. There is no difference between the sensitivities of AMP and CMP incorporation to inhibition by acridine orange and ethidium bromide. These experiments demonstrate clearly that tRNA is a site of inhibition by these fused ring heterocycles.

TABLE 3. Acridine Orange Pre-incubation

Preincubation mixture	Per cent inhibition
AO + sv-tRNA	83
AO + Enzyme	71
AO + sv-tRNA + enzyme	89

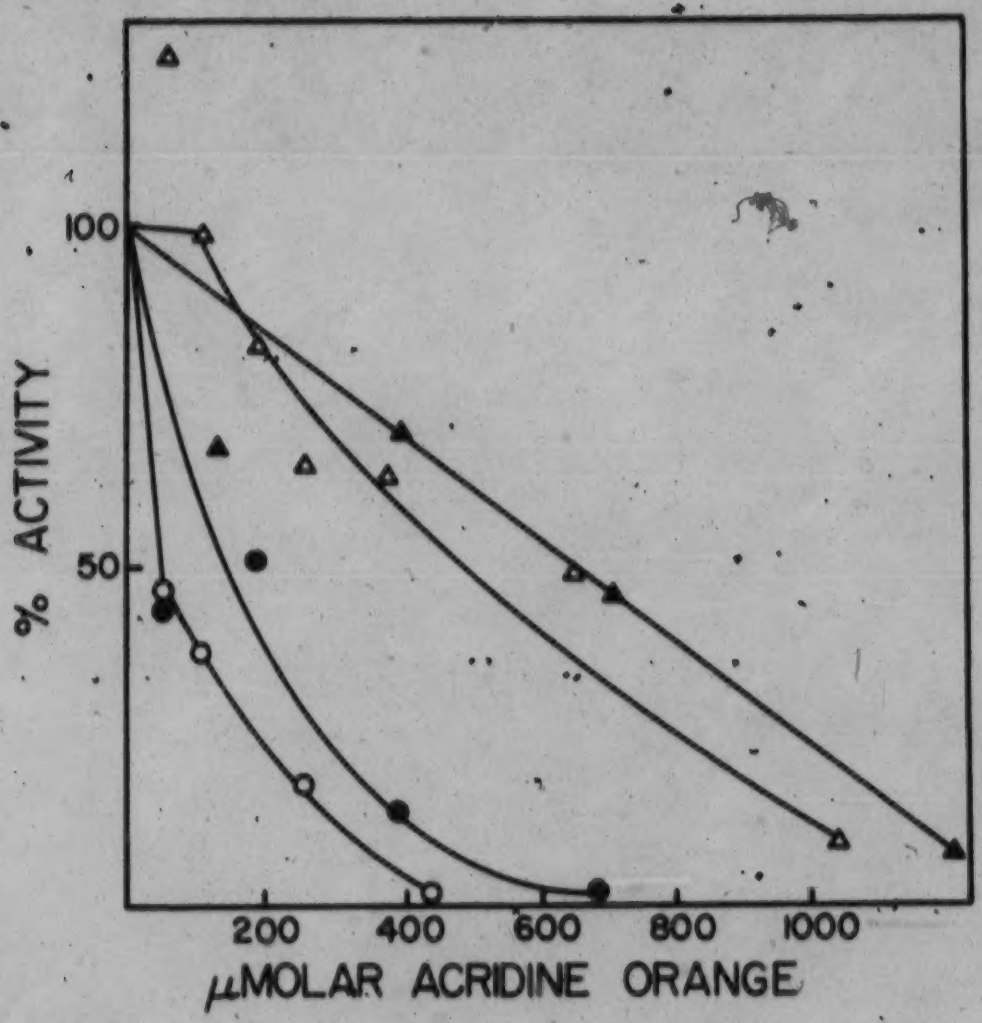


Figure 13: Effect of tRNA-concentration on inhibition by acridine orange. Assays were as in Materials and Methods, using DC fraction. 12.1 μg RNase-free enzyme was incubated with 58 μg (○) or 174 μg (△) tRNA-pXpCpC in the AMP-incorporating system with increasing concentrations of acridine orange. Alternatively, 12.1 μg enzyme was incubated with 61.2 μg (●) or 183.6 μg (▲) tRNA-pXpC in the CMP-incorporating system, also in the presence of varying dye concentrations.

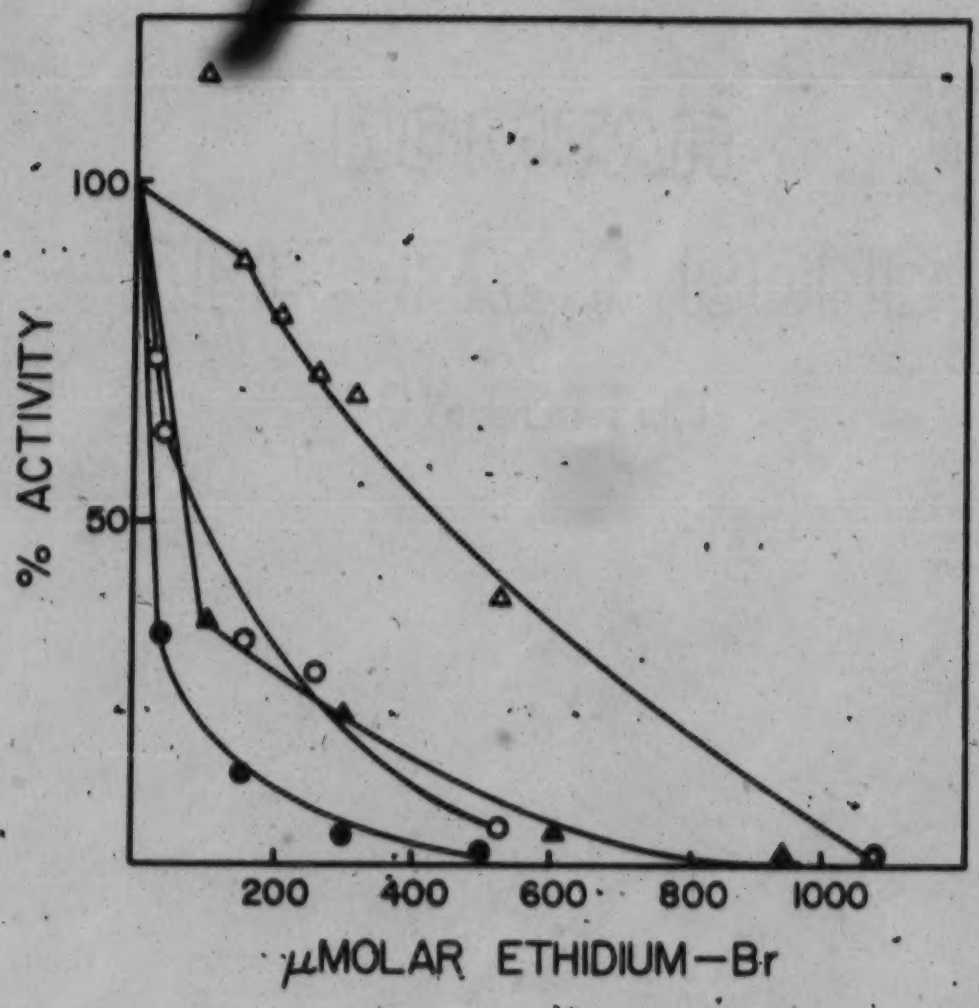


Figure 14: Effect of tRNA concentration on inhibition by ethidium bromide. Conditions were as given in Fig. 13. A 12.1 μg aliquot of DC fraction enzyme was incubated with 58 μg (○) or 174 μg (△) tRNA-pXpCpC in the AMP-incorporating system. The same amount of enzyme was incubated with 61.2 μg (●) or 183.6 μg (▲) tRNA-pXpC in the CMP-incorporating system.

Investigation of the Mode of Interaction

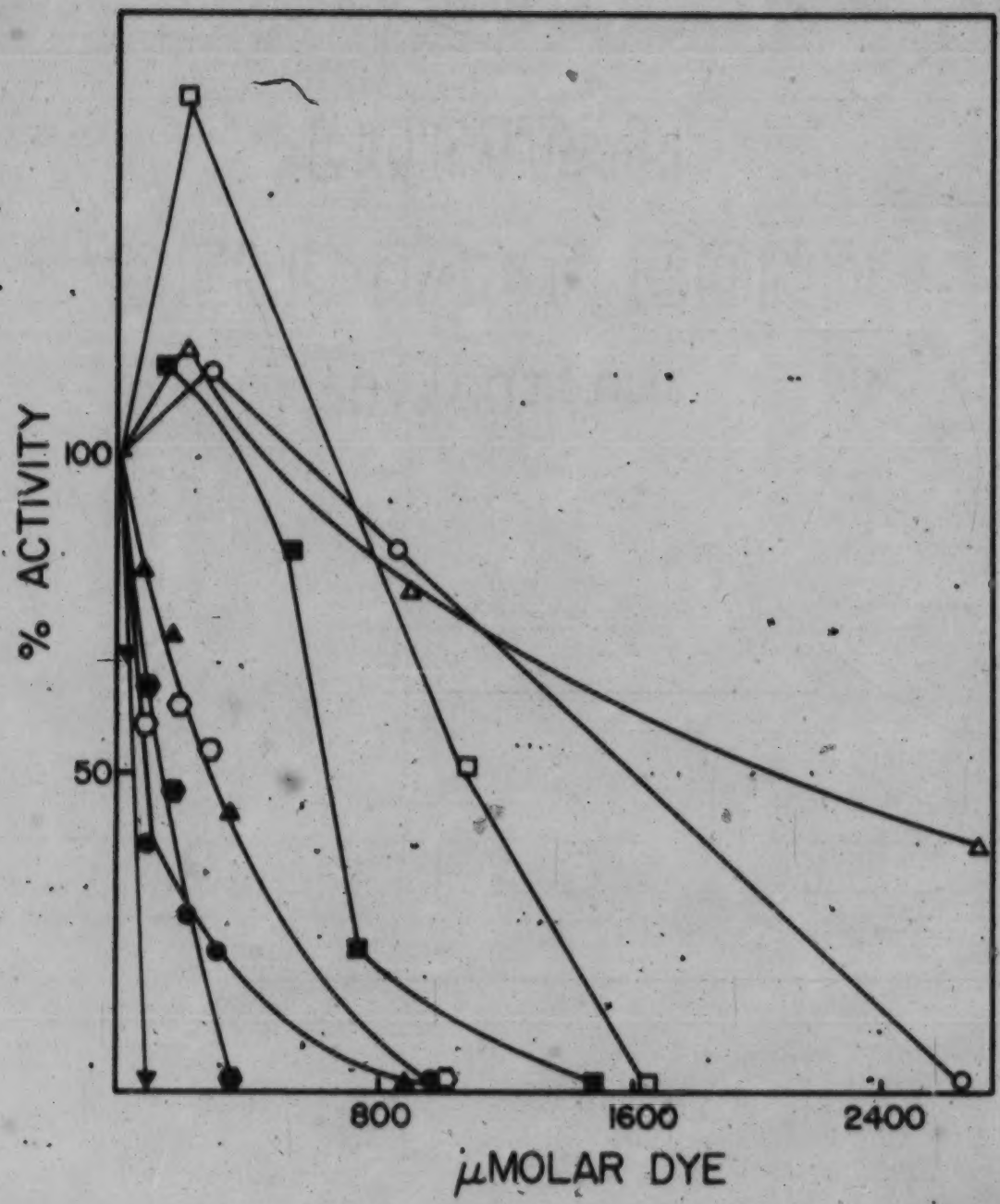
In order to determine whether inhibition could be caused by intercalation of dye molecules with the double helical regions of tRNA, Sephadex G25 chromatography was used. Free acridine orange was very strongly retained on the columns, but some dye molecules could be eluted with tRNA. Table 4 shows the acridine orange to tRNA molecule molar ratios of the load solutions and of the eluates. The molar ratios for the eluates were determined as described in Materials and Methods. At the lowest load solution ratio, there would be about 5% inhibition of the standard assay system. At the highest ratio, there would be about 60% inhibition. The eluates have ratios in the neighbourhood of 3 to 4, regardless of the ratio of the load solution. Three to 4 dye molecules is what can be expected to intercalate in the cloverleaf model of tRNA in the native conformation (92). The isolated s.v.tRNA-AO complex was examined for CMP incorporation. It was found that there is, in fact, no inhibition of CMP-accepting activity. Since the same value for the molar ratios is obtained regardless of the amount of dye loaded or of the extent of inhibition at that level, differential inhibition cannot be the result of intercalation.

The next step was to investigate the possibility of pseudo-polymer formation by dye. One way of approaching this is to examine the differences in inhibitory power for differently modified dyes. Using the standard assay system at pH 9, inhibition curves for several dyes were done, as shown in Fig. 15. Some of the dyes show an unexpected stimulation of activity in the low range of the concentration curve. At present, there is no explanation for this. It is obvious that all of the dyes do inhibit the reaction, at various

TABLE 4. Sephadex G25 Chromatography

Molar AO/tRNA ratio load solution	Molar AO/tRNA ratio eluate
9.0	4.05
29.8	3.10
53.7	3.67

Figure 15: Inhibition of transferase by different dyes. Assays were done as described in Materials and Methods. A 13.4 µg sample of DC fraction transferase was incubated with 92.8 µg s.v.tRNA in the CMP-incorporating system. Incorporation was measured in varying concentrations of the non-acridines: auramine-O (○), celestine blue (□), methylene blue (⬡) and fluorescein (△); and in the presence of the acridines: phosphine (■), acriflavine (●), 9-aminoacridine (▲), proflavine sulfate (⬢), and acridine yellow (▼).


% ACTIVITY
100
50
800
1600
2400
μMOLAR DYE

concentration ranges. To compare these ranges, I-50 values were taken from these graphs. The I-50 is the dye concentration at which 50% inhibition occurs. Table 5 gives these values for the acridines tested. These compounds are structurally very similar, except that the analogs differ in the extent and type of modification. Acridine yellow has four electron-donating substituents at the 2,7 and 3,6 positions. It is the most effective inhibitor of the acridines. The quaternary amine, acriflavine, is next. It is a better inhibitor than the otherwise similar proflavine sulfate, which has only 2 electron-donating groups. Acridine orange has the same number of electron donating groups as proflavine sulfate, yet it is a less efficient inhibitor. The difference between these two molecules is mostly in the size of the substituents; acridine orange has the large dimethyl amines, and proflavine the unsubstituted amines. The effect of the size of substituents is further demonstrated by the two-fold difference between the inhibitory power of 9-amino acridine and phosphine. The main structural difference between these two dyes is that phosphine has the bulky anilinium group at the 9-position while 9-amino acridine has the amine group.

Table 6 shows the I-50's for a series of non-acridines. In general, it can be seen that non-acridines are usually less potent inhibitors than acridines. Fluorescein is about the same size and shape as phosphine, yet its I-50 is three times as great. The crucial difference between them is in the charge distribution--fluorescein is negatively charged, while phosphine is positively charged.

Auramin-O differs from the other dyes in that it is not a fused ring system. However, its molecular dimensions are quite similar

TABLE 5. Inhibition by Acridine Analogs

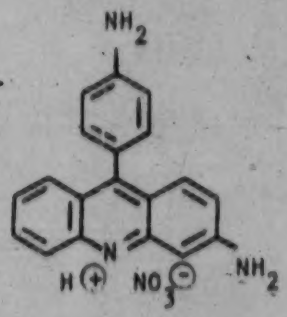
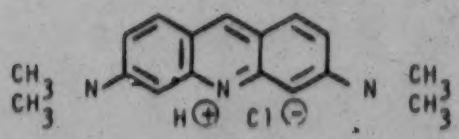
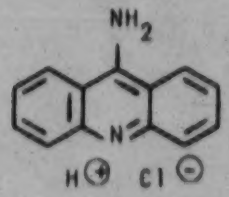
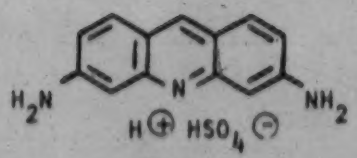
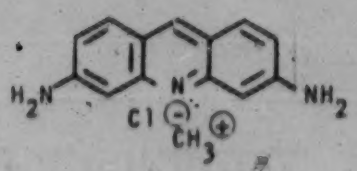
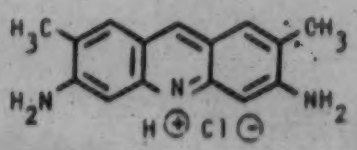
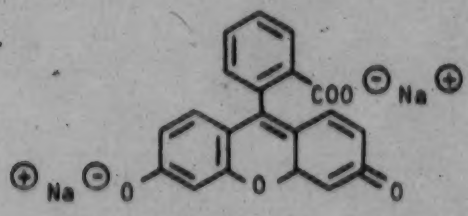
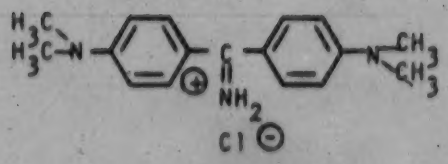
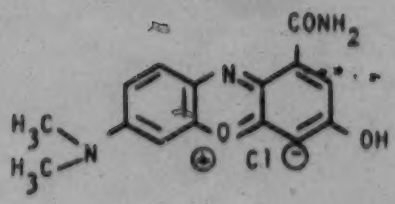
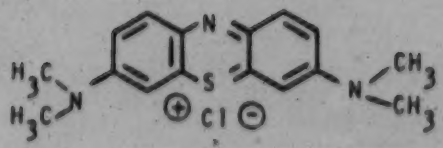
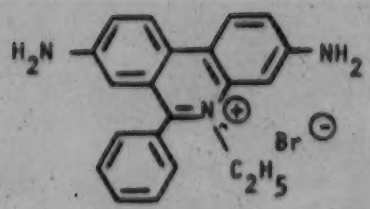
Name	Structure	I-50 (μM)
Phosphine		750
Acridine orange		334
9-Amino acridine		320
Proflavine sulfate		186
Acriflavine		96
Acridine yellow		50

TABLE 6. Inhibition by Non-acridines

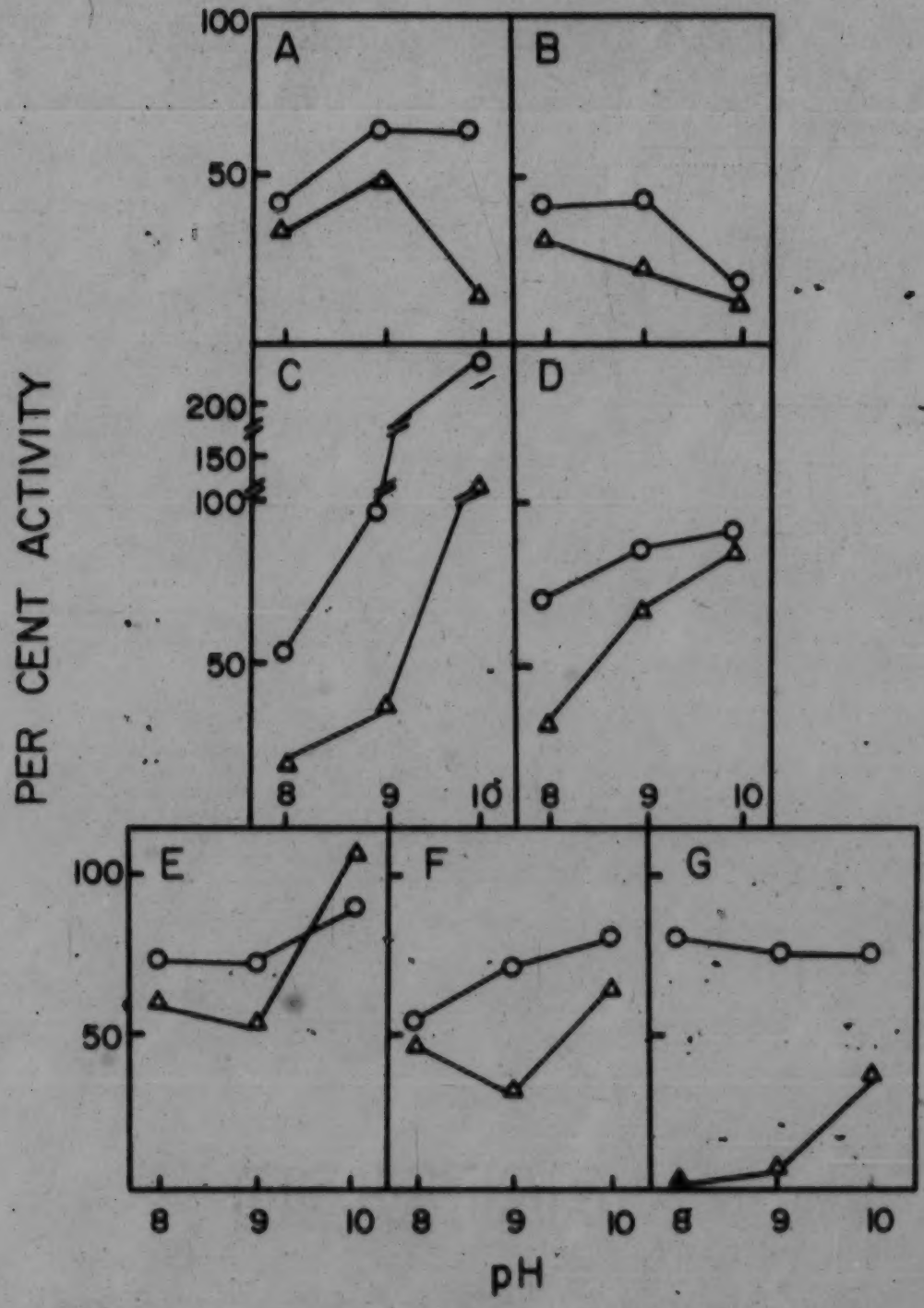
Name	Structure	I-50 (μM)
Fluorescein		2140
Auramine-O		1600
Celestine blue		1130
Methylene blue		294
Ethidium bromide		140

to acridine orange. Auramin-O does possess inhibitory power, but it is quite low. Celestine blue is an oxazole, and is not a particularly good inhibitor. Methylene blue is a thiazole, but aside from this modification, it is structurally identical to acridine orange. Not surprisingly, it inhibits in the same concentration range as does acridine orange. Ethidium bromide is a phenanthridine and a quaternary amine, and it has an I-50 which lies between those for acriflavine and proflavine sulfate.

pH Study

A pH study was undertaken to determine the extent of charge-involvement in inhibition. Since the acridine analogs all contain either tertiary amine groups with pK's around 9.5 to 10.5, or quaternary amines, it should be possible to manipulate the amount of positive charge on the ionizable dyes merely by changing the pH. The quaternary amines, acriflavine and ethidium bromide, are positively charged at all pH's. It is possible to detect fairly good transferase activity at pH 8, 9, and 10, so that a percentage activity can be calculated for a given amount of dye at each pH. If a positive charge is necessary for inhibition, the ionizable dyes should lose inhibitory power at pH 10, indicated by increased per cent activity at that pH. The quaternary amines should not show this effect. The pH curves are shown in Fig. 16. As expected, the ionizable acridines--phosphine, 9-aminoacridine, acridine orange, proflavine sulfate, and acridine yellow--show decreased inhibitory power at pH 10. The quantitative differences in the response to pH between these compounds can be related to differences in the pK of the amine group. In contrast to the ionizable dyes, the

Figure 16: Effect of pH on inhibition by dyes. Assays were performed as described in Materials and Methods except that pH was adjusted by use of appropriate glycine-NaOH buffers (final concentration, 0.05 M). In (A), 13.4 µg RNase-free transferase was incubated with 92.8 µg s.v.tRNA, in the presence of 83.2 µM (○) and 208 µM (△) acriflavine, in the CMP-incorporating system. In (B), incubation included 62.4 µM (○) and 124.8 µM (△) ethidium bromide; in (C), 558 µM (○) and 744 µM (△) phosphine; in (D), 272 µM (○) and 362 µM (△) 9-amino-acridine; in (E), 75.6 µM (○) and 151.2 µM (△) acridine orange; in (F), 91.6 µM (○) and 183.0 µM (△) proflavine sulfate and in (G), 34.5 µM (○) and 57.5 µM (△) acridine yellow.


PER CENT ACTIVITY
A
B
C
D
E
F
G
100
50
200
150
100
50
8
9
10
pH

quaternary amines do not lose inhibitory power at pH 10. Indeed, there seems to be greater inhibition at this pH, perhaps the result of some structural destabilization of the tRNA due to the high pH.

Effect of Fluorescein on Early Stages of the Reaction

In order to determine whether the inhibitory effects of the fused heterocycles were due to the first stage of tRNA-enzyme association, the effect of some acridines on tRNA binding to transferase was examined. Unfortunately, it was found that at pH 5.6 (the necessary pH for the binding assay), amine-containing compounds are precipitated, causing non-specific retention of labelled RNA on the filters. All of the fused heterocycles tested except fluorescein contain amine groups. For this reason, fluorescein was chosen as a model system to determine whether the fused heterocycles act at the level of initial binding. The effect of increasing concentrations of fluorescein on the binding of [^{3}H]CMP-tRNA and of [^{14}C]s.v.R17 RNA to transferase was therefore examined using the Millipore filtration method. The result is shown in Fig. 17: binding activity of both RNA species falls off as dye concentration increases. R17 RNA binding appears to be slightly more sensitive to the presence of fluorescein than tRNA binding. The reason for this may be that the complex between tRNA and enzyme may be more stable than that between R17 RNA and transferase. It has already been observed that the type of binding exhibited by these two polynucleates differs: tRNA binding is able to protect the enzyme from thermal inactivation, while R17 RNA binding is not. It can be seen from these data that the observed loss of incorporating activity in the presence of fluorescein may be attributed to decreased binding of tRNA substrate with enzyme.

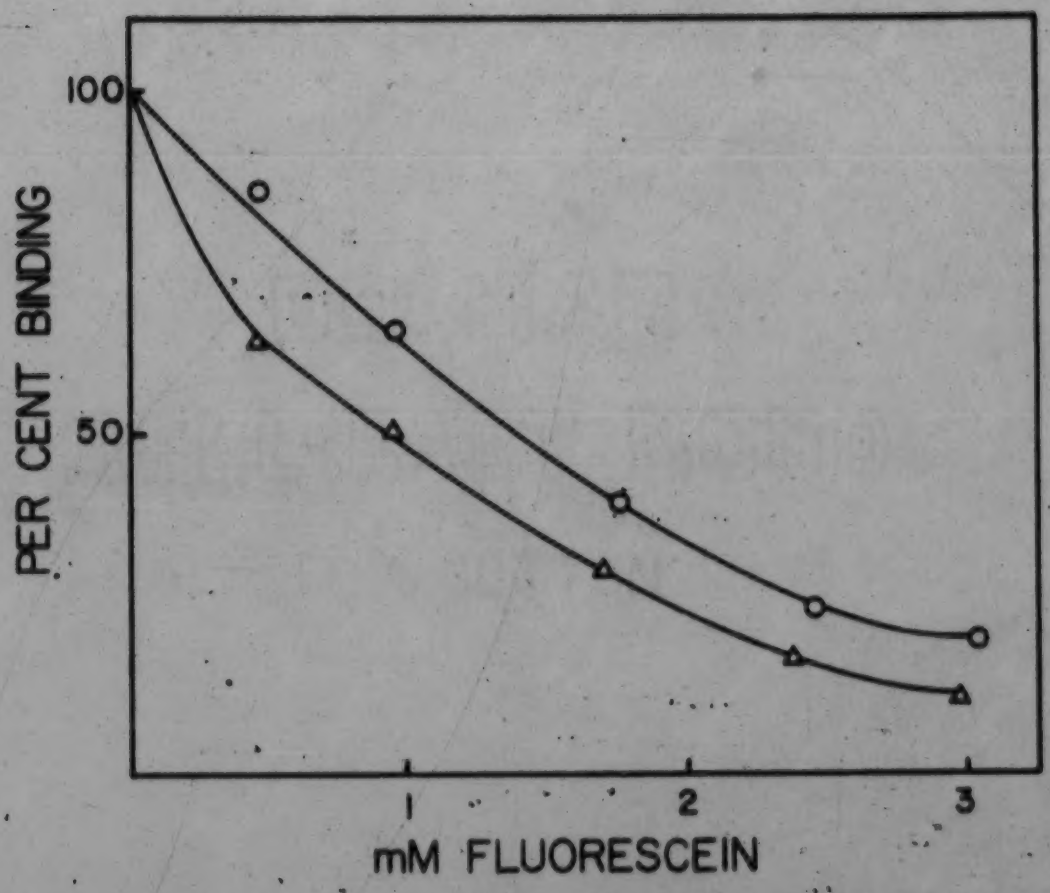


Figure 17: Effect of fluorescein on tRNA-enzyme and R17 RNA-enzyme binding. Binding was monitored by millipore filtration as outlined in Materials and Methods (Chapter 2). The incubation mixtures contained varying amounts of fluorescein. Controls at each dye concentration lacked enzyme. The amount of [^{3}H]CMP-tRNA (○) or [^{14}C]s.v.R17 RNA (△) bound was calculated by subtracting the amount of radioactivity retained on millipore filters in the absence of enzyme from the amount retained in the presence of transferase. The 100% level of binding in these experiments (that obtained in the absence of dye) was 24,000 c.p.m. /23 µg protein for tRNA and 37,000 c.p.m./23 µg protein for R17 RNA.

Fluorescence Study

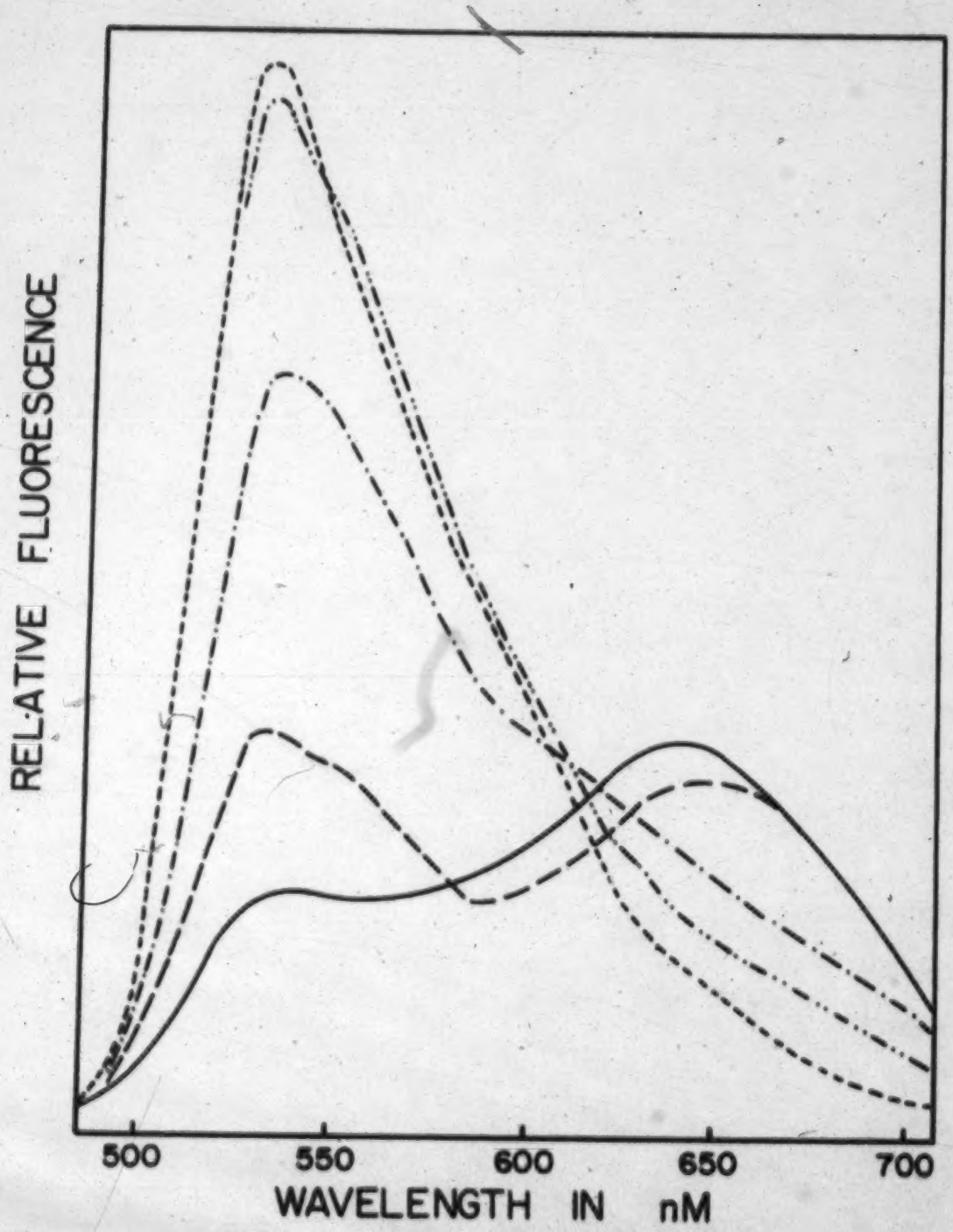
The comparison study (see above) indicates the fundamental necessity of the aromatic ring system in the process of inhibition. Fluorescence measurement is a good tool for the study of this aspect because it is so sensitive to changes in the environment of the aromatic portion of the dye molecules. Fig. 18 shows the fluorescence emission spectrum of acridine orange, in the presence of various polynucleates. In the absence of polynucleates, acridine orange has a fluorescence emission spectrum with a maximum at 535 nm. If RNA is added at a concentration higher than 10^{-5} M, a large shift in emission maximum to 650 nm is observed. In conjunction with this, the emission at 535 decreases. This is a differential effect--the greatest energy shift occurs for poly-A, then poly-G, with tRNA in the middle, then poly-C and poly-U.

Discussion

Inhibition of tRNA 3'-terminal nucleotidyl transferase by acridine and non-acridine dyes has been demonstrated. Of the reaction components, only tRNA concentration causes the inhibition level to change: if more tRNA is present, more dye is required to produce the same level of inhibition. This clearly indicates that tRNA is the site of inhibitory action. The question thus becomes: how does tRNA interact with dye molecules to produce a tRNA molecule that cannot accept CMP or AMP?

Two possibilities were investigated: (1) intercalation of dye molecules between successive bases in the RNA double helical sections, and (2) pseudopolymer formation of dye along the nucleic acid

Figure 18: Fluorescence emission spectra of AO in the presence of various polynucleates. Fluorescence was measured as given in Materials and Methods. Acridine orange at a concentration of 1.5×10^{-5} M was mixed with polynucleate at a concentration such that AO/RNA base = 1. Emission spectra are given for AO in the presence of poly-U (___), poly-C (_.._), tRNA (_._), poly-G (__ __), and poly-A (___). Incident wavelength was 300 nm.


RELATIVE FLUORESCENCE
500
550
600
650
700
WAVELENGTH IN nM

chain. In (2), the dye molecules self-stack with the positive charges oriented toward the nucleic acid chain, so that the force holding the nucleic acid and pseudopolymer together is ionic.

Intercalation can be excluded on the basis of the high concentration of dye required for inhibition, and on the basis of the gel filtration studies. Regardless of the acridine orange to tRNA molar ratios of the original mixture, 3 to 4 dye molecules were intercalated into each tRNA molecule. Urbanke et al have recently investigated by fluorescence methods the binding of ethidium bromide to different conformations of two purified tRNA species (92). They find that in the folded state (8°C), there are only three binding sites for ethidium bromide, this binding being characterized as intercalation. Yet at this level of AO, there is no inhibition of the transferase assay. The dye molecules that are involved in inhibition must form an association with tRNA which is less stable than intercalation.

Investigation of the possible nature of this interaction led to a study of variously modified dyes. In the acridine series, there is a clear correlation of inhibitory power with number of electron-donating substituents: the greater the number of electron-donating groups, the greater the inhibitory power of the dye (i.e. lower I-50). Electron-donating substituents would have two effects on these compounds. It is well known that electron donation raises the pK of ionizable groups on such molecules: the greater the number of electron-donating groups, the more basic the character of the amine. This effect is expected to be of minimal importance in differences in inhibiting power. However, since the variation in pK for these compounds

is not excessive (9.6 - 10.5). The second effect is that of increasing the stacking tendency: electron-donating substituents increase the tendency of heterocycles to interact by base stacking (81). Nakano and Igarashi (81) have found, for example, that for the formation of complexes with adenine in aqueous medium, the association constant for thymine (5-methyluracil) is almost double that for uracil. Thus this effect is likely of paramount importance in interpreting the I-50's of the acridine series. On this basis, acridine yellow would have the greatest stacking tendency, and it is the best inhibitor of the series. It is even better than the quaternary amine acriflavine, which might be expected to be the best inhibitor if the primary requisite for inhibition were a positive charge. Proflavine sulfate has two fewer electron-donating groups, and is less effective as an inhibitor. 9-Aminoacridine has only one such group in the more aliphatic position on the middle ring, and it is less efficient still.

One other factor emerges from the study of the acridine analogs. Acridine orange has the same number of electron-donating groups as proflavine sulfate, yet it is a less efficient inhibitor. The only difference between the two compounds is the presence of the bulky dimethyl-amines in acridine orange as opposed to the unsubstituted amines in proflavine sulfate. This size effect is further demonstrated by the two-fold greater inhibition by 9-aminoacridine over phosphine. Here the essential difference is that phosphine has far the bulkier group at the 9-position.

Thus, for the acridine series, two effects are obvious: first, inhibition is favoured by electron-donating substituents which

increase stacking tendency, and second, inhibition is disfavoured by bulky substitutions on the aromatic system of the dye.

The non-acridine series demonstrates a charge effect. Fluorescein is the same size and shape as phosphine, but it is only one-third as effective as an inhibitor . However, unlike phosphine, fluorescein is negatively charged. Thus a negative charge also disfavours inhibition. Another factor obtained from these data is that although the fused ring system is not essential for inhibition (auramin-O is able to inhibit), its absence results in low inhibitory power.

The fact that methylene blue inhibits in the same concentration range as acridine orange indicates that inhibition is not specific to charged amines. Methylene blue is a thiazole with the positive charge on the sulfur. Otherwise, it greatly resembles acridine orange. Nor is inhibition specific to linearly fused aromatic systems. Ethidium bromide is a phenanthridine and a quaternary amine, and its inhibitory concentration range is similar to the more effective acridines.

The pH data clearly demonstrate the importance of having a positively charged dye molecule. At pH 10 where more molecules will be in an un-ionized form than at pH 8 or 9, the ionizable acridines lose inhibitory power. The quaternary amines acriflavine and ethidium bromide do not lose inhibitory power throughout this range. Thus, clearly, a positive charge is important for inhibition. However, it is not sufficient, to judge from the low inhibiting action of auramin-O.

The fluorescence data highlight the part played by the aromatic ring system. Addition of polynucleate to acridine orange

solutions, when the polynucleate concentration is greater than 10^{-5} M, results in a shift in emission maximum from 535 to 650 nm. This shift is a differential effect--the greatest energy shift occurs for poly-A, then poly-G, with tRNA in the middle, then poly-C and poly-U. This order of the synthetic polynucleates corresponds to the order of self-stacking of the nucleic acid bases. If the association of dye with polynucleate, at this concentration range, is due to pseudopolymer formation by dye along the nucleic acid backbone, then there should be no differential effect among the different homopolynucleates, since there should be no direct interaction of the nucleic acid bases with the aromatic dye residues. The dye molecules should "see" only the phosphate backbone of the nucleates, and these should be virtually identical. However, it is obvious from the fluorometric results that the dye molecules do in fact "see" the individual bases of the RNA. Moreover, the interaction is such that the polynucleates made up of bases which have a greater tendency to self-stack also have a greater tendency to associate with dye molecules. This is very suggestive that the interaction between AO and tRNA primarily involves stacking between the dye and bases. This type of interaction would be compatible also with the finding that bulky substituents on the dye, especially in the position <u>para</u> to the positive charge, decrease the inhibitory power of that dye with respect to otherwise similar analogs, and with the finding that those dyes that are expected to have a greater tendency to stack are better inhibitors.

It has been found that fluorescein inhibits the first stage of the reaction, causing a reduction in the binding of RNA to enzyme. tRNA binding is more resistant to the presence of dye than is R17 RNA

binding. R17 RNA binds non-specifically to transferase. The binding is sufficient to cause inhibition of the transferase reaction with tRNA, but not sufficient to result in incorporation of nucleoside monophosphate into R17 RNA or to protect the enzyme from thermal inactivation. The greater relative sensitivity of R17 RNA over tRNA, for binding in the presence of dye, may be due to weaker binding of the large phage RNA and transferase. Since the polycyclic dyes interact with nucleic acid via a semi-specific process, probably involving base-stacking, it is not surprising to find that the initial phase of the reaction, i.e. binding of tRNA to transferase is the one that proves sensitive to the dye.

Therefore, although intercalation is not involved in inhibition, the alternative hypothesis of some sort of dye pseudo-polymer formation appears not to be completely valid either. Two phenomena have been demonstrated: one is ionic and the other hydrophobic in nature. The results suggest a two-stage interaction: the first is ionically mediated. The positive charge of the dye interacts with the negative phosphate charges on the polynucleate. Then the aromatic portion of the dye is brought into stacking interaction with the aromatic bases of the RNA. The result is a tRNA species which cannot accept AMP or CMP in the tRNA 3'-terminal nucleotidyl transferase system, and which cannot even bind to transferase.

CHAPTER 5

OTHER PROBES OF tRNA RECOGNITION BY TRANSFERASE*

Introduction

The results of Chapter 4 indicate that fused-ring heterocycles inhibit the transferase reaction by interacting with the transfer RNA substrate. This interaction prevents binding to the enzyme and in this manner inhibits catalytic action by transferase.

The search now turns to other probes of the system. One compound that could be expected to alter the behaviour of the protein component is aurin tricarboxylic acid (ATA). It has been shown that this substance effectively inhibits several systems involving protein-RNA interactions. These include aminoacyl-tRNA synthesis (93), protein synthesis (94-96), and mRNA-ribosome interactions (95,96). The effective concentration of dye varies depending on the system and on the concentrations of reaction components within the system reported. However, there is general agreement that the inhibitions occur through association of ATA with protein. An advantage that ATA possesses as a probe of enzyme action is the fact that several differently-modified analogs are available. The basic unit (aurin) is a triphenylmethane group, to which various substituents are attached in different ring positions. By comparing the inhibitory actior of the analogs, it may

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*It should be noted at this point that for this chapter, the transferase preparation contained trace amounts of aminoacyl-tRNA synthetase, but no RNase.

be possible to determine what aspects of ATA structure produce inhibition, which in turn may throw some light on the structure of the catalytic site on the enzyme.

In spite of this expectation, other characteristics of ATA might lead one to speculate other causes for inhibition. For example, as a dye, ATA forms a magnesium "lake" by complexing with the metal ions (97). This "lake" is what is applied to certain types of cloth to make the dye fix, .to attach the dye permanently to the cloth. The transferase system has a very strict magnesium requirement (see Chapter 3). If ATA inhibits, and if a complex is formed, then the cause of inhibition may be removal of the magnesium necessary for the enzymic reaction.

The results indicate that the triphenylmethanes do inhibit the tRNA nucleotidyl transferase system, and that this inhibition does not involve either complex salt formation with magnesium ion or interaction with tRNA. The site of action is the enzyme. The data permit speculation that ATA itself may attack a nucleotide-recognition site. The binding study indicates that this site is one involving the tRNA nucleotide(s).

Other probes, whose action is not so well understood as that of ATA, are the polyamines. The presence of these compounds in the cells of most organisms is widely known. They seem to be present ir fairly high concentration (2-5 mM), and appear to function in various ways. See, for review, Introduction to the Polyamines (98). It has been observed for instance that spermine, spermidine, cadaverine, and Mg^{+2} partially protect protoplasts against thermal lysis (99). Also spermine, putrescine, and Mg^{+2} are specific in promoting the induction

of premature chromosome condensation of the interphase nuclei of hybrid HeLa cells (100). Another type of action is that of counteracting various types of inhibition, e.g. mescaline destabilization of brain cortex ribosomes (101), pactamycin-inhibition of the initiation of protein synthesis (102), and streptomycin inhibition of leucine incorporation into protein (E. coli system) (103). A third type of action is that of stimulating or activating several types of activities, e.g. binding of ribosomes to endoplasmic reticulum (104), amino-acyl tRNA synthesis (105), amino acyl-tRNA binding to ribosomes (106-108), protein synthesis (109,110), and tRNA-methylase activity (111). It is known that polyamines bind very strongly to DNA (112), so that some of their effects can be seen to be due to this factor. In other cases, the concensus seems to be that they function mainly as replacers of Mg^{+2}. The mechanism is not well understood; the molecules are polycations and are present in vivo in about the concentrations which produce the various effects that have been observed.

In the transferase system, it was found that, at low concentrations, polyamines caused varying levels of stimulation depending on the amount of Mg^{+2} present. At higher concentrations, inhibition of transferase activity was observed. The stimulatory effect appears to be additive: even at optimal Mg^{+2} concentration, there is marked stimulation by the polyamines, especially spermine. This excludes the possibility that polyamines stimulate transferase activity solely by replacement of magnesium. The true cause of stimulation is not at present known. However, inhibition at higher concentrations has been found to be due to precipitation of the tRNA

substrate, preventing association of this species to the enzyme, as determined by thermal inactivation studies.

Results

Effect of Aurin Derivatives on the Incorporation Reaction

Concentration profiles for the inhibition of CMP incorporation into s.v.tRNA were obtained for several dyes, under the standard assay conditions outlined in Chapter 4. From these curves, I-50 values for the triphenylmethane dyes were determined as before (see Chapter 4). These results are summarized in Table 7. ATA inhibits at a very low concentration (3 μM) and is clearly much more effective than even the next-best inhibitor in this series (40-fold difference in I-50). It is also more effective than the acridine series, although some of the analogs of ATA inhibit in a concentration range close to that of the acridines.

Investigation of Mode of Action of ATA

To determine what reaction component(s) interact with ATA, it was decided to examine several aspects of the transferase system. Since, in dye chemistry, the aurins are known as metal ion complexers (105), it could be speculated that their inhibitory action is related to the magnesium requirement of the transferase reaction. However, the inhibitory concentration of ATA (3 μM) is far too low for it to be acting through this mechanism (Mg^{+2} concentration is 5 mM).

As a preliminary step to discovering whether enzyme or tRNA were the site of inhibition, the effect of addition order was examined. Table 8 shows the result of adding ATA after s.v.tRNA, but before

TABLE 7. Inhibition by Aurins

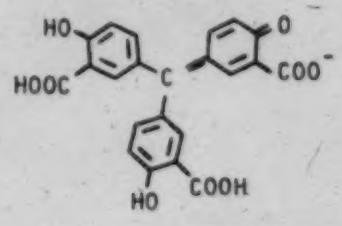
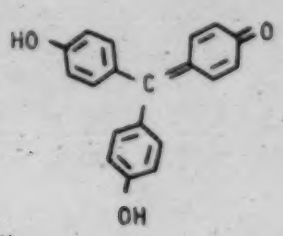
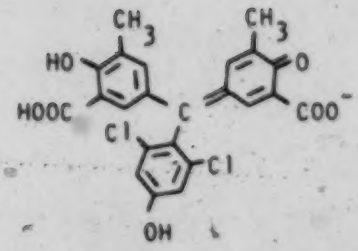
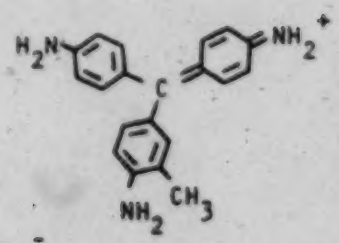
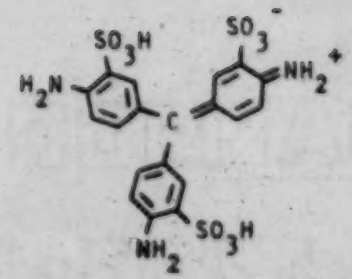
Name	Structure	I-50 (μM)
ATA		3
Aurin		110
Azure blue B		320
Fuchsin basic		157
Fuchsin acid		680

TABLE 8. Effect of Addition Order on ATA Inhibition

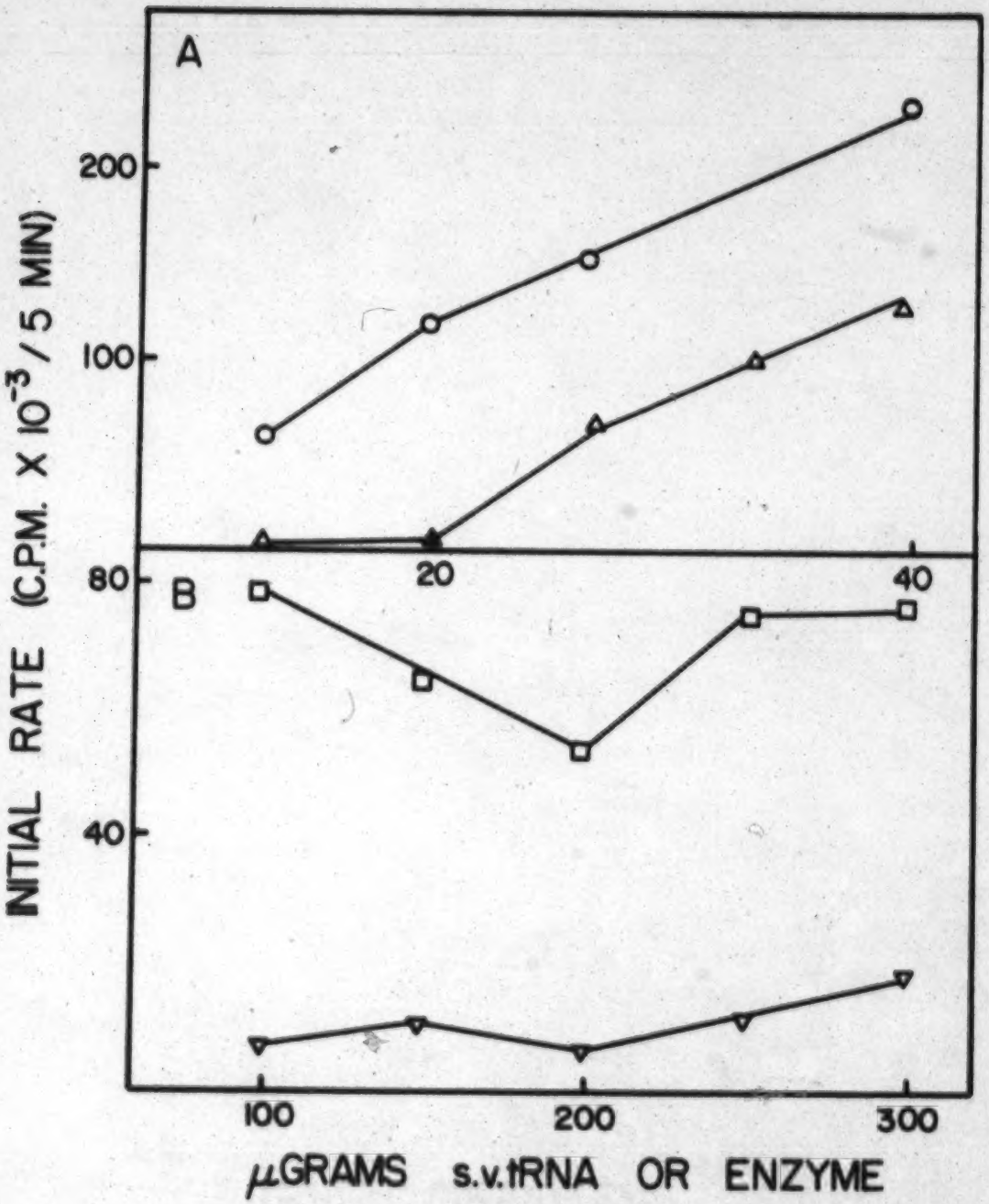
Addition Order at 3 μM ATA	% Activity
(1) s.v.tRNA → ATA → enzyme	55.0
	56.7
(2) s.v.tRNA → enzyme → ATA	83.4
	80.5

enzyme, and of adding it after enzyme (when s.v.tRNA is already present). Inhibition is greater when enzyme is added last. This could suggest either that tRNA is the site of inhibition or that the presence of tRNA protects the enzyme from ATA action. To clarify this issue, the effect of changing the concentration of s.v.tRNA and of enzyme was examined. This result is shown in Fig. 19. Increasing the tRNA concentration in the presence of 10 μM ATA results in no recovery of incorporating activity. It should be noted that at 100 μg s.v.tRNA/0.5 ml reaction mixture, the system is already saturated for that component., On the other hand, increasing the enzyme concentration under the same conditions results in substantial recovery of activity after 20 μg enzyme/0.5 ml reaction mixture. Moreover, the recovery occurs so that the curve (+ATA) is parallel to the curve (-ATA). Exactly analogous results were obtained when less ATA was used (< 100% inhibition point under the standard conditions). These data demonstrate that the enzyme is indeed the site of inhibition.

Effect of Aurin Derivatives on Early Stages of the Reaction

The effect of triphenylmethane dyes on the binding of nucleic acid to transferase was amenable to study since three of the tested compounds contain no amines and hence do not precipitate at pH 5.6: aurin, azure blue B, and aurin tricarboxylic acid (ATA). Fig. 20 shows the effect of increasing concentrations of aurin and azure blue B on the binding of [^{3}H]CMP-tRNA to enzyme. Binding declines steadily as dye concentration increases. With this in mind, it was decided to examine ATA effect on the binding of both [^{14}C]s.v.R17 RNA and [^{3}H]CMP-tRNA to the enzyme. If there were a significant difference, that might

Figure 19: Effect of enzyme and tRNA concentration on inhibition by ATA. Incorporation was measured in the absence of ATA (○, □) and in the presence of 10 μM ATA (△, ▽). In graph A, enzyme concentration was increased from 14 μg to 40 μg per 0.5 ml reaction mixture. In graph B, s.v.tRNA concentration was increased from 100 μg to 300 μg per 0.5 ml reaction mixture.


A
B
200
100
80
40
20
40
100
200
300
INITIAL RATE (C.P.M. X 10^{-3} / 5 MIN)
μGRAMS s.v.tRNA OR ENZYME

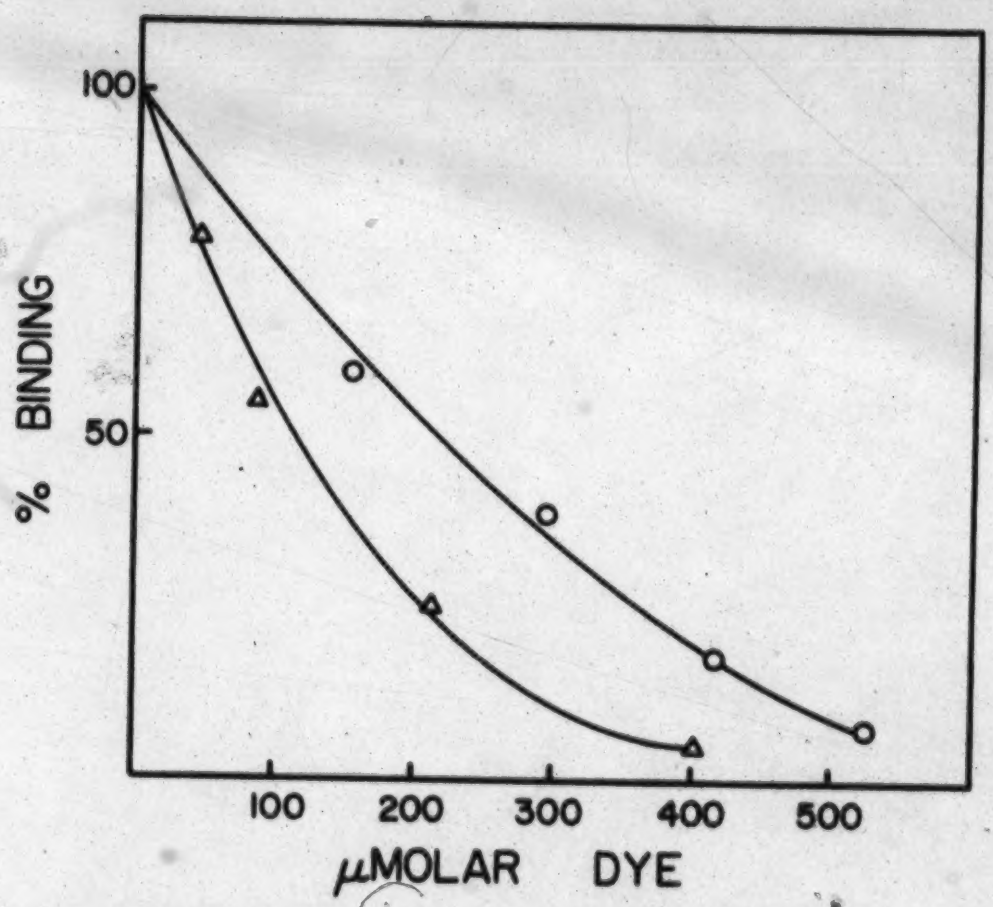


Figure 20: Effect of aurin and azure blue B on tRNA-enzyme binding. Binding of transferase to [^{3}H]CMP-tRNA was monitored as described in Materials and Methods, in the presence of varying amounts of aurin (Δ) and azure blue B (O). The amount of label bound was calculated by subtracting the amount of radioactivity retained on millipore filters in the absence of enzyme from the amount retained in the presence of transferase. The 100% level of binding in these experiments (that obtained in the absence of dye) was 24,000 c.p.m./ 23 μg protein.

give some information as to which type of binding was being inhibited: the non-specific type shown by both R17 RNA and tRNA, or the specific type shown only by tRNA. As can be seen in Fig. 21, there is no significant difference in the I-50 of binding for the two polynucleates, indicating that the former type of binding is being inhibited.

Effect of Polyamines on Transferase in the Absence of Mg^{+2}

The polyamines investigated were: spermine $H_2N(CH_2)_3NH(CH_2)_4NH(CH_2)_3NH_2$, spermidine $H_2N(CH_2)_4NH(CH_2)_3NH_2$, and putrescine $H_2N(CH_2)_5NH_2$. Fig. 22 shows that all of the polyamines are capable of some stimulation of transferase activity when Mg^{+2} is absent from the system (enzyme was desalted by Sephadex G25 chromatography). 100% activity is the activity at zero polyamine in the presence of 5.2 mM magnesium. Of the polyamines, spermine stimulates at the lowest concentration, with a sharp maximum at 1 mM. Spermidine has a broader maximum from 1 - 3 mM, while putrescine has a maximum that is broader yet (2 - 10 mM). Spermine and spermidine both inhibit the reaction at 10 mM, the inhibition being greater for spermine. Putrescine does not show this severity of inhibition, although activation does decrease slightly by 10 mM putrescine.

Effect of Polyamines in the Presence of Mg^{+2}

In the presence of magnesium, stimulation by polyamines was observed. The effect appeared to be additive. Fig. 23 shows the concentration profiles for spermine in the presence of 0, 0.2 mM, and 5.2 mM magnesium. There is a slight shifting of maximum, from 1 mM

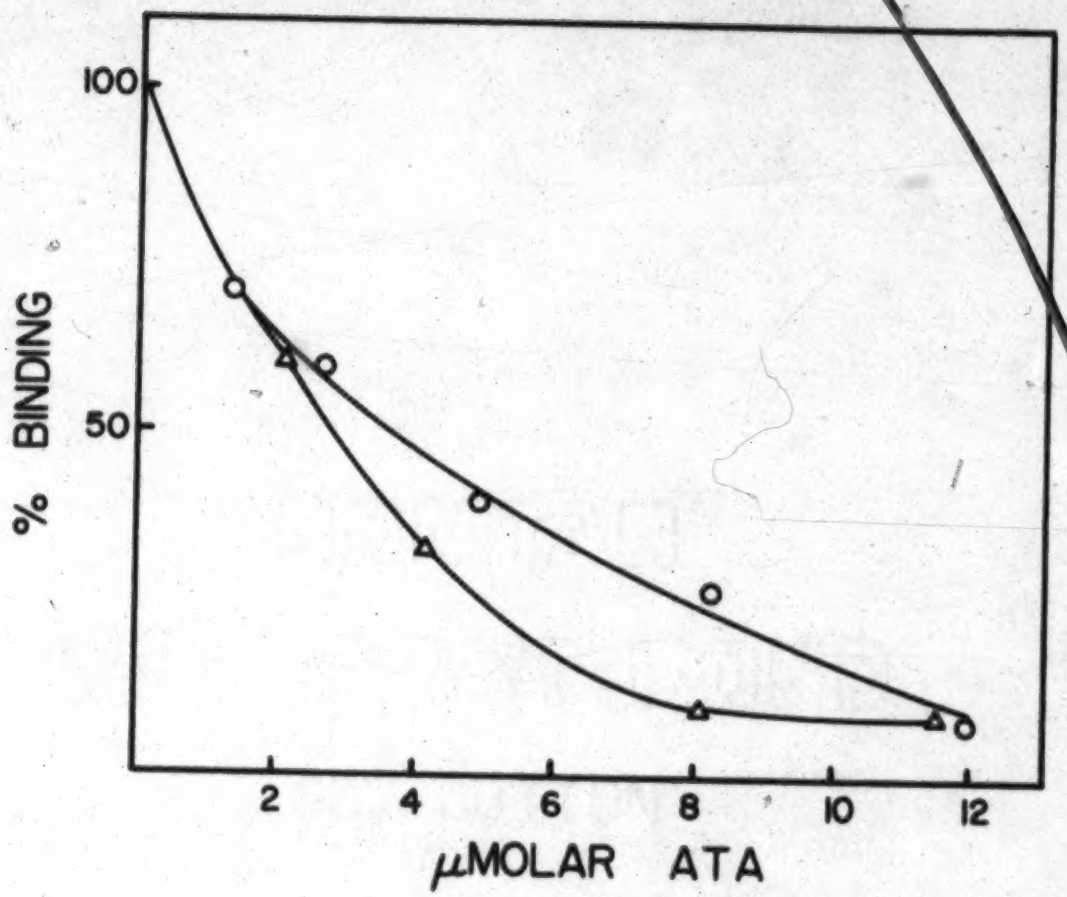


Figure 21: Effect of ATA on tRNA-enzyme and R17 RNA-enzyme binding. Binding of transferase to [^{3}H]CMP-tRNA (O) and to [^{14}C]s.v.R17 RNA (Δ) was measured as given in Fig. 20, in the presence of varying concentrations of ATA. The 100% level of binding was 37,000 c.p.m./23 μg protein for R17 RNA.

Figure 22: Effect of polyamines on transferase in the absence of Mg^{+2}. Assays were done as in Materials and Methods. Enzyme was desalted to remove Mg^{++} by passing through Sephadex G-25. On the same day, the desalted enzyme was used for these assays. Enzyme that exists for over a few hours in the absence of salt loses activity and responds anomalously to polyamines. Incorporation of [^{3}H]CMP into s.v.tRNA was measured in the presence of spermine (○), spermidine (□), and putrescine (△). 100% activity is the activity when 5 mM Mg^{++} is added to a reaction mixture containing no polyamine.

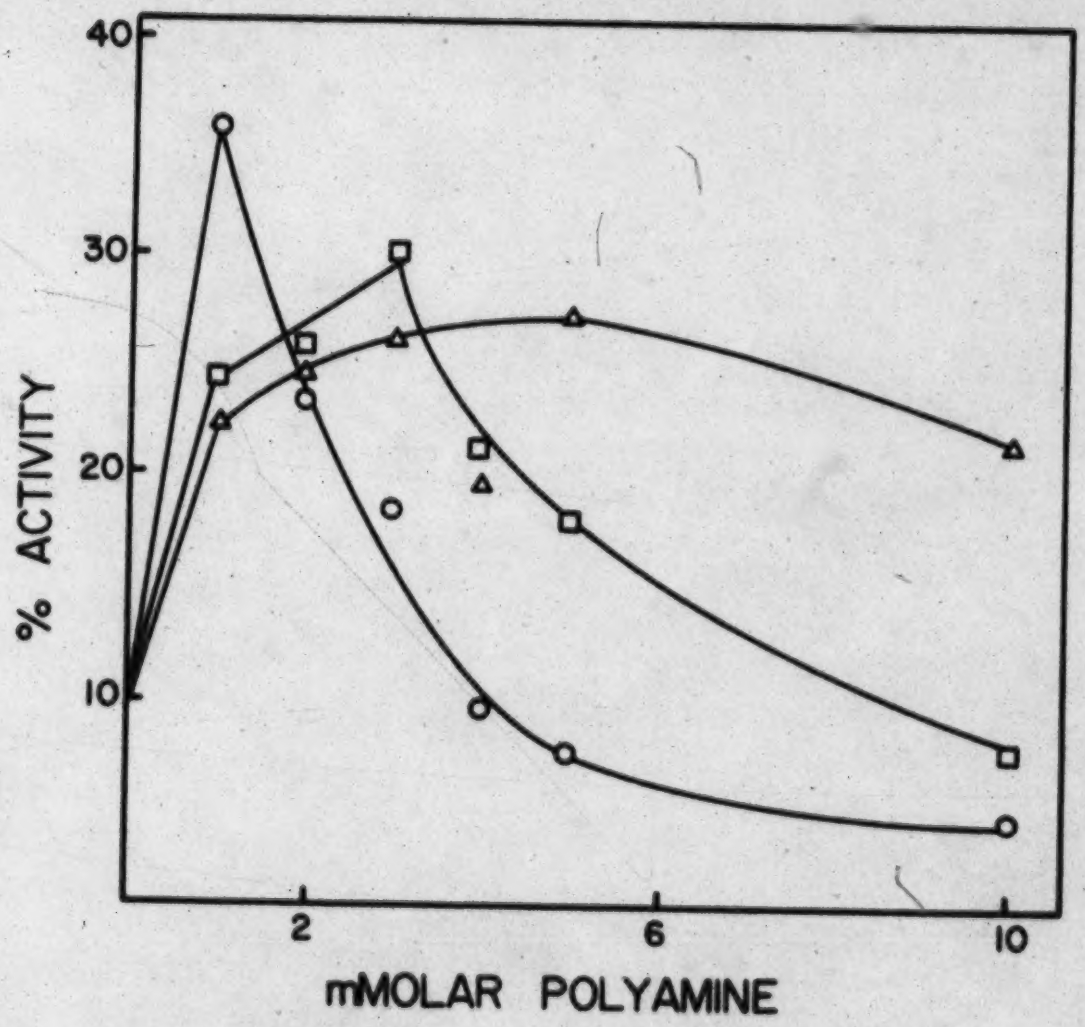
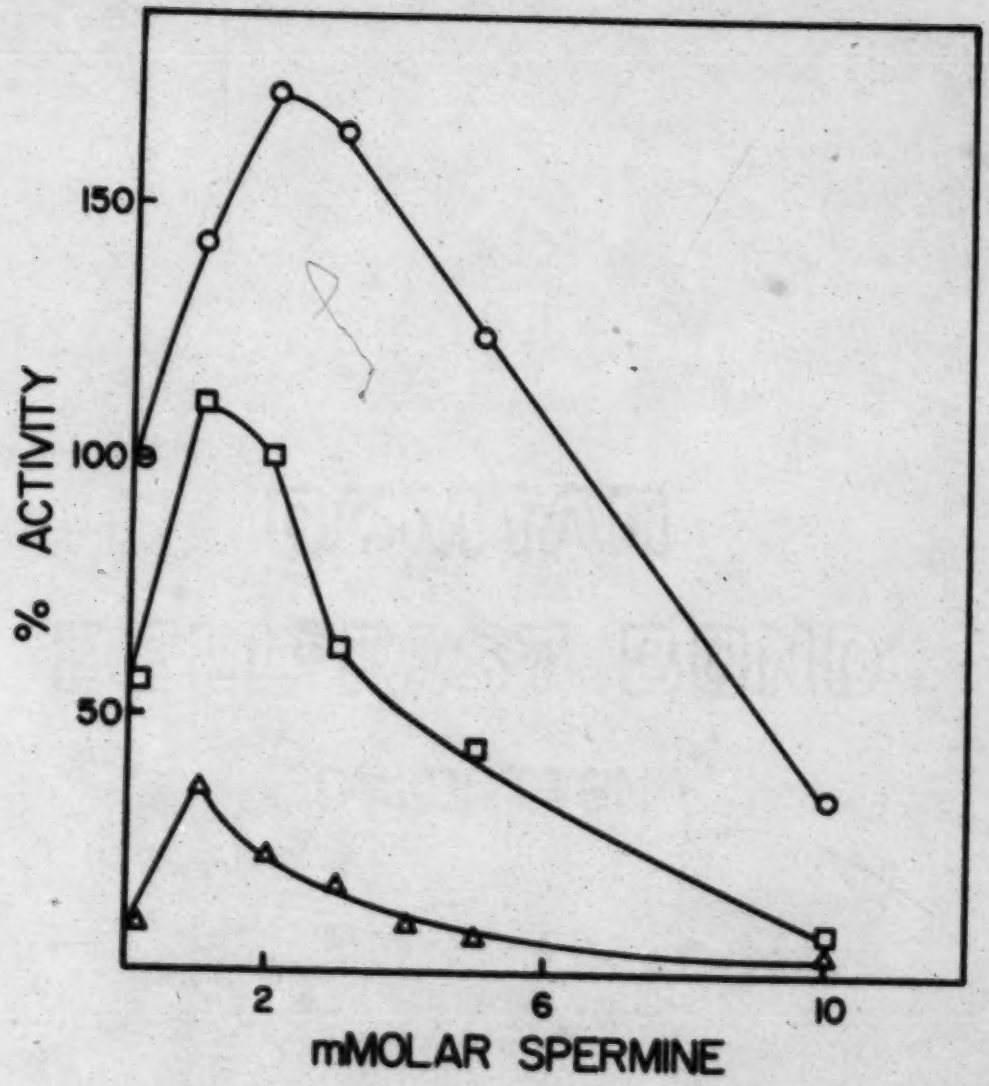

40
30
20
10
% ACTIVITY
2
6
10
mMOLAR POLYAMINE

Figure 23: Effect of spermine on transferase at varying Mg^{++} concentrations. Assays were performed as in Fig. 22. Only for the zero Mg^{++} curve was the enzyme desalted by Sephadex G-25. It was not necessary for the others. Incorporation of ^{3}H-CMP into s.v.tRNA was measured at 0 mM Mg^{++} (△), 0.2 mM Mg^{++} (□), and at 5.2 mM Mg^{++} (○) at spermine concentrations up to 10 mM.


150
100
50
% ACTIVITY
2
6
10
mMOLAR SPERMINE

when no magnesium is present to 2 mM when 5.2 mM Mg^{+2} is present. In each case, at an appropriate spermine concentration, there is increased activity observed over that obtained with only magnesium present. The 100% level for all curves is the extent of incorporation in the absence of polyamine, but in the presence of 5.2 mM Mg^{+2}. It is also clear that there is inhibition at the higher concentrations of spermine. Fig. 24 shows these data for putrescine. The stimulatory effect is much more diffuse for putrescine than for spermine, and at 5.2 mM magnesium, the effect seems to be destroyed. However, it can still be seen that at sub-optimal Mg^{+2} concentrations (0 mM and 0.2 mM), the effect of putrescine is additive.

Investigation of the Mode of Action of Spermine

In an effort to see if polyamines act primarily on enzyme or on tRNA, a simple experiment was done, altering the s.v.tRNA to enzyme ratio. Two conditions were used: (a) a ratio of s.v.tRNA/enzyme of 37.2 on a molar basis (taking the molecular weight of enzyme to be 56,000, estimated from Sephadex chromatography), and (b) a ratio of s.v.tRNA/enzyme of 9.3. Using these conditions, a concentration profile of spermine at 5.2 mM magnesium was done as shown in Fig. 25. There is no discernible difference in the profiles, and the maximum is again 2 mM spermine, as it was in the standard condition of Fig. 23 where the ratio of s.v.tRNA to enzyme is 18.6. The difference in extent of stimulation shown here and that shown in Fig. 23 is the result of the age of the polyamine solution: as it grows older, stimulation decreases. If spermine acts specifically on enzyme, the lower tRNA/enzyme ratio should cause a shift in maximum to a

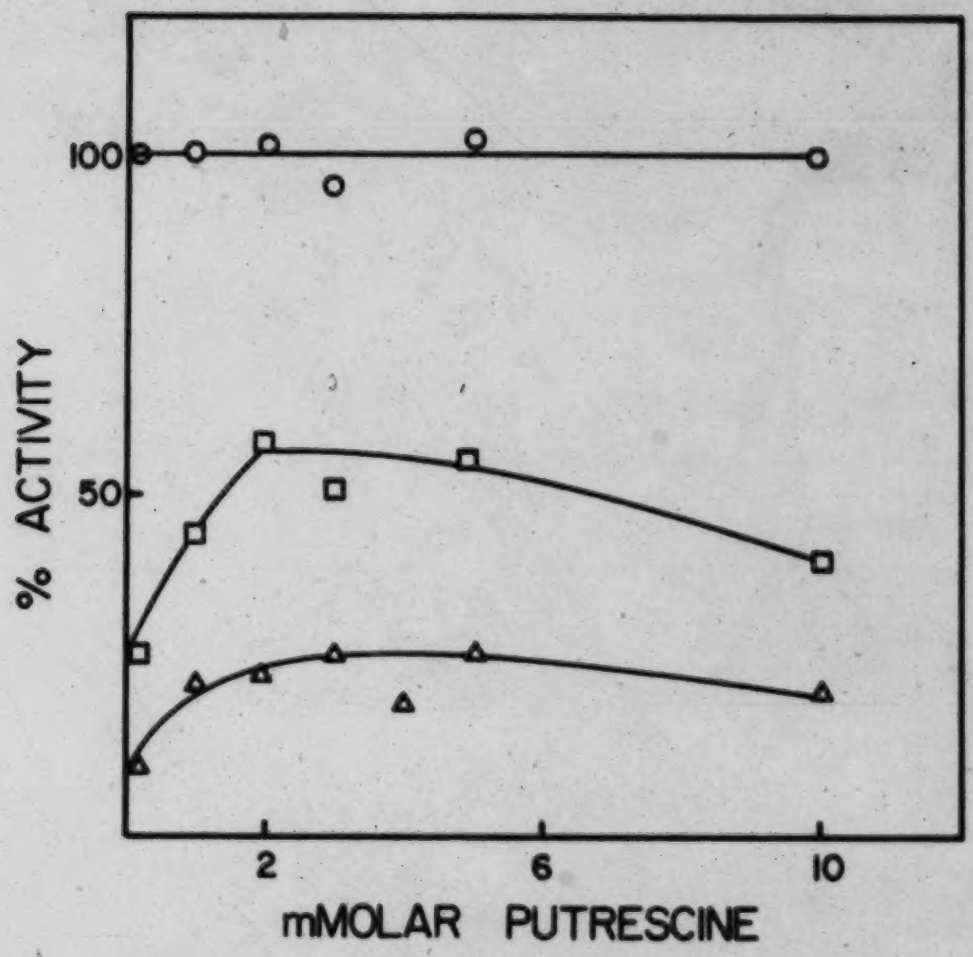


Figure 24: Effect of putrescine on transferase at varying Mg^{++} concentrations. Assays were done as described for Fig. 23. Incorporation of [^{3}H]-CMP into s.v.tRNA was measured in the presence of 0 mM Mg^{++} (Δ), 0.2 mM Mg^{++} (□), and 5.2 mM Mg^{++} (○) at putrescine concentrations up to 10 mM.

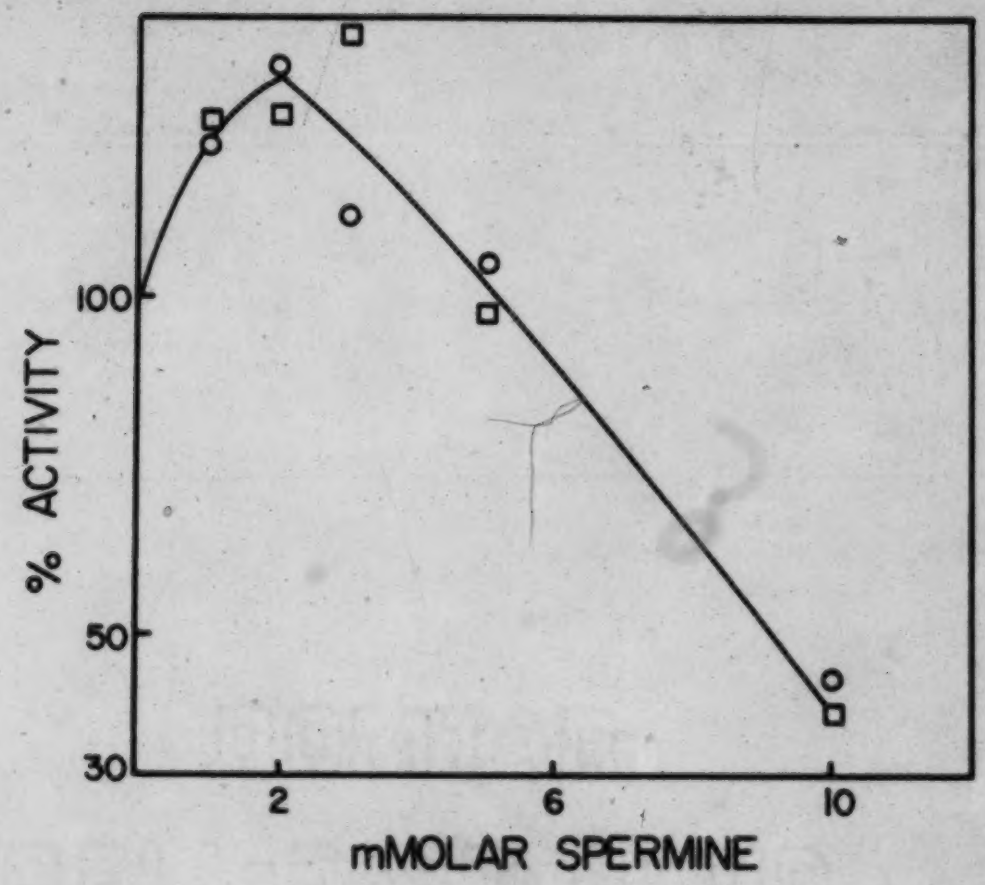


Figure 25: Effect of s.v.tRNA to enzyme ratio on the spermine activation profile for transferase. Incorporation of [^{3}H]-CMP into s.v.tRNA was measured for spermine concentrations up to 10 mM, when the s.v.tRNA to enzyme ratio was 37.2 (O) on a molar basis and when it was 9.3 (□) on a molar basis.

higher spermine concentration. Conversely, if tRNA were a specific site, the higher ratio should cause a shift. Neither of these conditions prevail, indicating that there is no specific effect on either enzyme or RNA.

Although there was no specific effect found, it was thought that there might be an effect on thermal inactivation of transferase. From Chapter 3, it can be seen that transferase is readily inactivated at 50°C in the absence of materials that stabilize it (e.g. tRNA). Transferase was pre-incubated at 4° and 50°C for 20 min in the presence of varying amounts of spermine. Activity was then assayed by adjusting the spermine concentration in each tube to 2 mM. 100% activity at a given spermine pre-incubation concentration was the activity of the control pre-incubated at 4°C. As can be seen in Fig. 26, there is some slight protection as spermine concentration increases; however, the profile does not correspond to the profile of stimulation and inhibition of activity as seen in Fig. 23. It seems very likely that this protection is due to a non-specific salt effect (it had been found that it was necessary to remove salts from those enzyme preparations containing High Salt Buffer before carrying out thermal inactivation studies: Chapter 3).

Effect of Spermine on Early Stages of the Reaction

In order to determine whether the stimulatory and/or inhibitory action of polyamines was due to the first stage of tRNA-enzyme association, the effect of spermine on tRNA binding to enzyme was examined using Millipore filtration. Unfortunately, amine-containing compounds are precipitated at pH 5.6, causing extensive and erratic

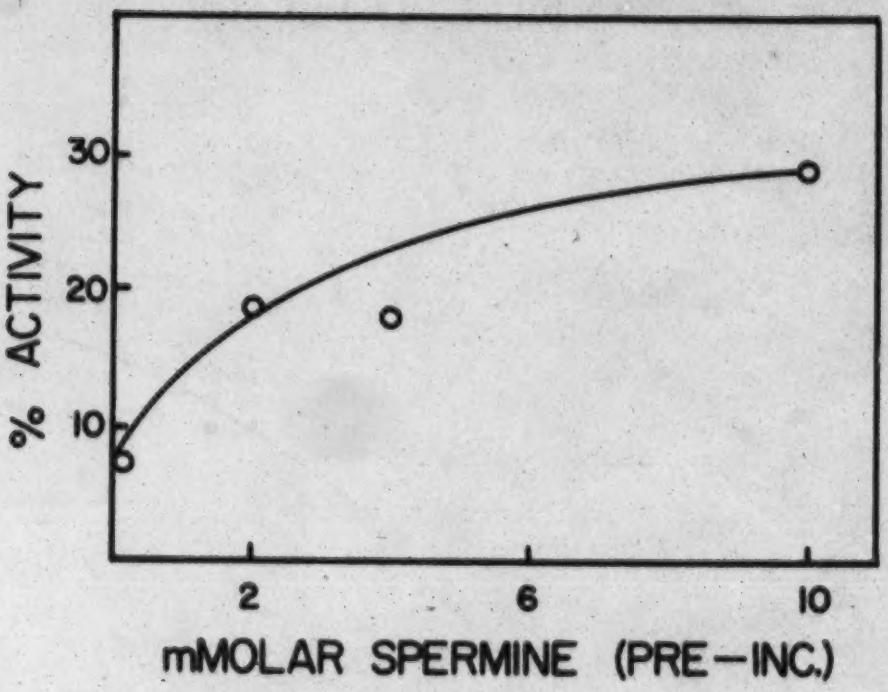


Figure 26: Effect of spermine on thermal inactivation of transferase. Duplicate tubes containing transferase and varying concentrations of spermine in 50 mM Glycine-NaOH (pH 9), 5 mM $MgCl_2$, and 1 mM βME were pre-incubated for 20 min at 4°C or at 50°C. Spermine concentration was then adjusted to 2 mM, the other reaction components were added, and activity remaining was measured as [^{3}H]CMP incorporation into s.v.tRNA at 37°C. Per cent activity is calculated taking the controls at 4°C pre-incubation temperature as 100%.

non-specific retention of labelled RNA on the filters. Hence, it was impossible to monitor the first stage of tRNA-enzyme interaction by this method. Other technical difficulties made gel filtration methods also unsuitable, even though higher pH's could be used.

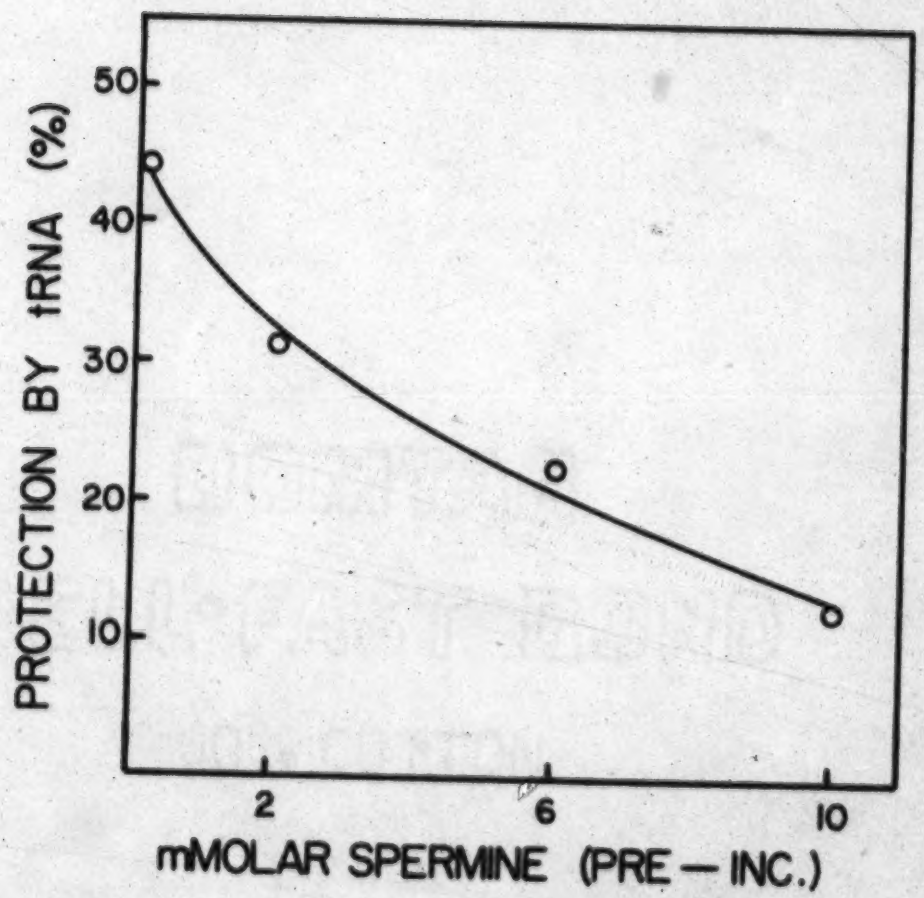
Thus, it was decided to examine thermal inactivation of transferase, thereby monitoring all stages of association between tRNA and enzyme prior to actual incorporation. It is clear that spermine itself confers a certain amount of thermal stability on the enzyme. The protection due to polyamine had to be eliminated via experimental design in order to determine what effect spermine has on the interaction between transfer RNA and enzyme. This was done by adding controls at each spermine concentration. The protection due to tRNA alone was then determined by the following reactions:

$$\% \text{ activity} = \frac{\text{initial rate (pre-incubation at 50°C)}}{\text{initial rate (pre-incubation at 0°C)}} \cdot 100$$

Protection due to tRNA = (% activity for pre-incubation in the presence of tRNA) − (% activity for pre-incubation in the absence of tRNA)

for each spermine concentration tested. The result is shown in Fig. 27. There is no stimulation of protection at any concentration tested; rather, protection due to tRNA is seen to decline steadily as spermine concentration increases. Moreover, a white precipitate was observed in those tubes containing tRNA and 6 or 10 mM spermine. A subsequent test of precipitation of tRNA by spermine showed that 65.1% of the A_{260} in the pre-incubation mixture was precipitated at 10 mM spermine. Thus, the loss of ability of tRNA to protect the enzyme can

Figure 27: Effect of spermine on tRNA protection of transferase from thermal inactivation. Thermal inactivation was performed by pre-incubation of 12 µg of enzyme in the presence or absence of tRNA and/or spermine, in a total volume of 50 µl, for 20 min at 50°C. Following this step, the standard assay system, in which the spermine concentrations had been adjusted to 2 mM, was used to determine activity remaining. In both the pre-incubation and assay mixtures, the pH was 9.0. At each spermine concentration, there were four samples, two without tRNA and two containing 96 µg s.v.tRNA. One of each duplicate was pre-incubated at 50°C for 20 min, while the control was maintained on ice. The % activity at each spermine concentration in the absence of tRNA was calculated from a comparison of the initial rate of the 50° sample with the initial rate of the control kept at 4°C. A similar calculation was carried out to obtain % activity at each spermine concentration in the presence of tRNA. The protection due to tRNA alone is the difference between these values.

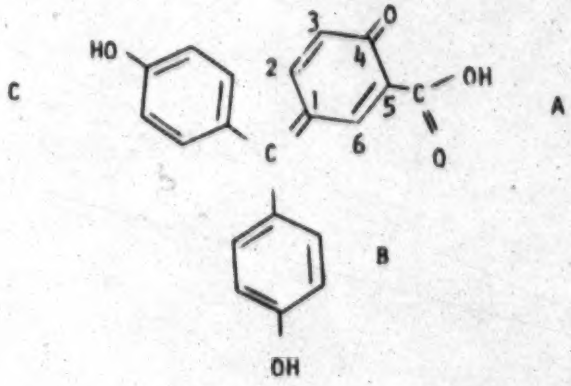

PROTECTION BY tRNA (%)
50
40
30
20
10
2
6
10
mMOLAR SPERMINE (PRE — INC.)

be seen to be due to loss of access to the enzyme. The decline in protection by tRNA explains the inhibitory action of spermine at high concentrations. It is also clear that the stimulatory action at low concentrations cannot be due to any stage of tRNA-enzyme association prior to interaction with the nucleoside triphosphate substrate. The cause of this stimulation is still a mystery, although it is now clear that the stimulation does not affect the recognition of tRNA by transferase.

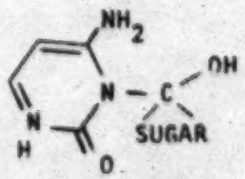
Discussion

It has been demonstrated that ATA and its analogs inhibit the tRNA 3'-terminal nucleotidyl transferase reaction. The triphenylmethanes that were studied can be divided into two groups--those that resemble ATA, having no charge or a net negative charge, and those that resemble fuchsin basic, having a positive charge on the molecule. ATA, aurin, and azure blue B belong to the first group; fuchsin basic and fuchsin acid to the second group. At physiological pH, fuchsin basic is positively charged and will interact with nucleic acid. Fuchsin acid has zwitterion character. These two compounds are effective in the range of the acridines (see Chapter 4), and may inhibit the transferase reaction by a different mechanism than ATA. It is not the intention of this study to produce more compounds that interact with RNA, so the cationic triphenylmethanes are included merely for interest. The group of aurins that resemble ATA are not expected to interact with RNA; in fact, it is found that ATA interacts specifically with enzyme. Examining structural differences among these compounds (Table 7), it can be seen that loss

of the carboxyl groups at C5, with loss of the negative charge, renders aurin a much less efficient inhibitor than ATA (40-fold difference in I-50). On the other hand, azure blue B possesses the carboxyl groups, but it has in addition, a bulky methyl substituent at C3:



This bulky substitution drastically reduces the inhibitory power: azure blue B is 100 times less effective than ATA. This contrasts markedly with the effect of methyl substitution on the aromatic rings of the acridine series (Chapter 4), where an increase in inhibitory power occurred due to increased stacking tendency of the dyes. Considering the ring A in the structure of the triphenylmethanes given above, it can be seen that bulky substitution at C3 and loss of the carboxyl group at C5 reduce the structural similarity between that part of the triphenylmethane molecule and nucleotides, for example cytidine:



The addition order experiment shows less inhibition of transferase if enzyme and s.v.tRNA are present together, prior to addition of ATA than if enzyme is added last (which was the normal order used for the concentration curves). This suggests that tRNA may partially protect the enzyme from the action of ATA. It may be that ATA associates with the enzyme at a site normally occupied by tRNA. In fact, the binding study indicates that aurin, ATA, and azure blue B cause inhibition of the binding of tRNA to transferase. In addition, R17 RNA binding to transferase is sensitive to ATA at about the same dye concentration as tRNA binding, this binding being monitored for similar µg quantities of each polynucleate. This suggests that ATA interacts with the non-specific nucleic acid binding site(s) on transferase (as discussed in Chapter 3), rather than only with the specific site that tRNA alone is able to occupy. The inhibition of binding then prevents catalysis by transferase. Thus, understanding the mode of interaction of transferase and small cyclic compounds like ATA may lead to a determination of the mechanism of tRNA recognition.

For the polyamines, the picture is less clear. It is obvious that these compounds both stimulate and inhibit activity in the incorporation reaction, depending on the concentration. The order of effectiveness of polyamines is spermine>spermidine>putrescine: the more cationic species are more stimulatory. The effects are additive, suggesting that polyamines in this system do not function as mere replacers of Mg^{+2}. There has not been a specific site of action found, but it has been observed that there is a salt-type effect on thermal inactivation. Spermine shows only one effect on the initial

stages of tRNA-enzyme interaction. Thermal protection of the enzyme by tRNA declines steadily as spermine concentration in the pre-incubation mixtures increases. There is no increased protective effect at 2 mM spermine. The stimulatory effect thus does not involve an influence on tRNA-enzyme association, prior to the binding of the nucleoside triphosphate substrate. The inhibitory effect seems to be the result of physical separation of the tRNA substrate and transferase, since it has been found that high concentrations of spermine cause tRNA to precipitate.

Thus, the polyamines represent compounds which have considerable effect on the transferase system, but whose function is only partially determined. Future interest will no doubt be directed to determining the cause of the stimulatory effect and its relation, if any, to the process of tRNA recognition by transferase. For the aurins, the compound of importance is ATA. It inhibits the incorporation reaction at very low concentrations by interaction with transferase itself. Moreover, the inhibition occurs at the first stage of the reaction, i.e. binding of tRNA. The specificity of this compound makes it attractive as a probe of the enzymic recognition site for transfer RNA.

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